GENERAL PHYSIOLOGY

Founded by Jacques Loeb

EDITORS
W. J. CROZIER JOHN H. NORTHROP
W. J. V. OSTERHOUT

VOLUME 13, No. 1 SEPTEMBER 20, 1929

307

PUBLISHED BIMONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1929, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

as second-class matter November 25, 1918, at the Post Office at Baltimore, Md., under the act of March 3, 1879

Made in the United States of America

CONTENTS

LILLIE, RALPH S. Resemblances between the electromotor variations of rhythmically reacting living and non-living systems. Plates 1 and 2 1
HARVEY, E. NEWTON. A preliminary study of the reducing intensity of luminous bacteria 13
NORTHROP, JOHN H. Unequal distribution of ions in a collodion cell 21
SHOUP, CHARLES S. The respiration of luminous bacteria and the effect of oxygen tension upon oxygen consumption 27
OSTERHOUT, W. J. V., and HARRIS, E. S. Note on the nature of the current of injury in tissues 47
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. I 57
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. II 81

The Journal of General Physiology is issued bimonthly, one volume of about 600 pages appearing in a year. Contributions should be sent to the editors of *The Journal of General Physiology*, York Avenue and 66th Street, New York, N. Y. The papers should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Charts larger than 10 x 12 inches should be accompanied by photographic reproductions, or by tracings which can be folded to the size of the manuscript for convenience in mailing. Papers will be printed as soon as possible after acceptance, but *not necessarily in the order in which they are received for publication.* Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or York Avenue and 66th Street, New York, N. Y

CONTENTS

ANSON, M. L., and MIRSKY, A. E. Protein coagulation and its reversal. The preparation of completely coagulated hemoglobin 121
MIRSKY, A. E., and ANSON, M. L. Protein coagulation and its reversal. The reversal of the coagulation of hemoglobin 133
KRUEGER, A. P., and TAMADA, H. T. The preparation of relatively pure bacteriophage 145
EMERSON, ROBERT. Measurements of the metabolism of two protozoans 153
EMERSON, ROBERT. Some properties of the pigment of *Blepharisma* 159
EMERSON, ROBERT. On the behavior of nickel carbonate in relation to photosynthesis 163
ABRAMSON, HAROLD A. Electrokinetic phenomena. I. The adsorption of serum proteins by quartz and paraffin oil 169
RAHN, OTTO. The size of bacteria as the cause of the logarithmic order of death 179
DAMON, E. B. Dissimilarity of inner and outer protoplasmic surfaces in *Valonia*. II 207
BLINKS, L. R. Protoplasmic potentials in *Halicystis* 223
GATES, FREDERICK L. A study of the bactericidal action of ultra violet light. I. The reaction to monochromatic radiations 231
GATES, FREDERICK L. A study of the bactericidal action of ultra violet light. II. The effect of various environmental factors and conditions 249
OSTERHOUT, W. J. V. The kinetics of penetration. I. Equations for the entrance of electrolytes 261

The Journal of General Physiology is issued bimonthly, one volume of about 600 pages appearing in a year. Contributions should be sent to the editors of *The Journal of General Physiology*, York Avenue and 66th Street, New York, N. Y. The papers should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Charts larger than 10 x 12 inches should be accompanied by photographic reproductions, or by tracings which can be folded to the size of the manuscript for convenience in mailing. Papers will be printed as soon as possible after acceptance, but not necessarily in the order in which they are received for publication. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or York Avenue and 66th Street, New York, N. Y.

RESEMBLANCES BETWEEN THE ELECTROMOTOR VARIATIONS OF RHYTHMICALLY REACTING LIVING AND NON-LIVING SYSTEMS

By RALPH S. LILLIE

(From the Laboratory of General Physiology, University of Chicago, Chicago)

PLATES 1 AND 2

(Accepted for publication, May 3, 1929)

It is well known that under certain conditions the reaction of iron wire with nitric acid exhibits an automatic rhythm of sometimes remarkable regularity, consisting in an alternation of active and passive periods.[1] In a recent study of this phenomenon[2] I have found the most regular rhythms in soft iron wire of low carbon content. Wire drawn from iron prepared by electrolysis of pure solutions of iron salts[3] and the pure commercial soft iron known as Armco[4] are especially favorable. In hard steel wire (piano wire) the tendency to rhythm is slight, and any rhythm shown is local and irregular. In general the essential conditions for a regular and rapid rhythm (*e.g.*, 50 to 100 per minute), involving all parts of a wire of some length (10 cm. and more), may be defined as follows: (1) The acid must be of sufficient strength (> 55 v.%), such that the passive state is one of stable equilibrium; the wire then reverts automatically to the passive state after each activation (spontaneous repassivation); (2) the wire must transmit states of activation readily and quickly; (3) it must recover its transmissivity rapidly after activation; and (4)

[1] For a comprehensive account of rhythmical reactions in metals and in inorganic systems in general *cf.* the monograph by Kremann, R., "Die periodischen Erscheinungen in der Chemie," *Ahrens Sammlung chemischer und chemisch-technischer Vorträge*, 1913, 19, p. 289; also the recent book by Hedges and Myers, "The Problem of Physico-chemical Periodicity," London, E. Arnold & Co., 1926.

[2] Lillie, R. S., *Archivio di Scienze Biologiche*, 1928, 11, 102, and *Science*, 1928, 67, 593.

[3] Kindly furnished by the U. S. Bureau of Standards, Washington.

[4] American Rolling Mill Co. This iron contains 99.84% iron and less than 0.02% carbon, according to analyses furnished by the Company.

to those found in steel wire, but the absolute rate of the process is much greater.

The rhythm is most readily demonstrated as follows. A short length of clean bright Armco wire (*e.g.*, 2 mm. thick, 1 to several cm. long) is placed in a flat-bottom vessel containing nitric acid of 60 to 80 v.% concentration.[7] Usually a rhythmical reaction starts at once; the metallic surface shows at regular intervals an alternation between a steel bright and a dull lustre; on closer examination it is seen that the effervescence is confined to the dark periods, while during the bright periods the metal is non-reactive (passive). The number of cycles per minute varies usually between 40 and 100 or more, increasing with the concentration of acid and the temperature. The relative duration of the passive phase in each cycle increases with the strength of acid; in the weaker acid the appearance is that of a bright flash passing at intervals over the dark effervescent surface of the metal. If the acid be left unstirred, the rhythm soon becomes irregular and passes over into a continuous effervescence; but with stirring a regular rhythm may last indefinitely, *i.e.*, until most of the wire is dissolved.[8]

Experiment shows that under these conditions the presence of the continually active or controlling region depends on the contact of the metal with the glass. At some local area of contact the diffusion of reaction products and acid is retarded and the acid soon becomes too weak to repassivate the iron; the reaction then becomes continuous, *i.e.*, a permanently active or anodal area is established. A wire freely suspended in acid by thin glass filaments is non-rhythmical; when activated it shows a single reaction and immediately becomes again passive and remains so. Rhythm, however, can readily be induced by locally interfering with diffusion or otherwise maintaining a permanent local activity.[9] The method finally adopted was to insert one end of the suspended wire into the interior of a narrow glass tube for a few millimeters. When such a wire is activated a few times (by touching

[7] Volumes of HNO_3, C. P., Sp. Gr. 1.42, in 100 volumes of solution.

[8] For further details *cf.* Lillie, R. S., *loc. cit.*[2]

[9] *E.g.*, continual contact of copper, zinc or other metal, anodal in relation to passive iron, will induce rhythm; but such rhythms are irregular because of the difficulty of maintaining constant conditions.

a single localized region must be present in which the reaction of the metal with the acid is continuous; this permanently active region may be compared with the nodal or pace-making region of the heart; it furnishes a constant activating influence to which the rest of the wire responds as soon as it has recovered sufficiently. From this controlling region waves of activation travel along the wire at intervals determined by the duration of the non-transmissive or "refractory" period which immediately follows each activation. In the rhythmically reacting wire, as in the heart, the regularity of the rhythm thus depends on the uniformity of the process of recovery, while the coördination or synchronization of activity in the different regions depends on transmission from a central or controlling region.

Since activation and transmission in passive iron are known to depend on the alteration or breakdown[5] of the passivating surface film by local cathodic reduction, it is clear that the special properties of the film differ in different kinds of iron and are determined, in some manner not yet understood, by the special composition of the metallic surface. The film formed on steel wires during repassivation is highly resistant to reduction by the current of the local active-passive circuit; hence transmission of activation for more than a short distance is at first impossible. It is only after some minutes (in 70 v.% HNO_3 at 20°) that the wire regains the ability to transmit activation waves to an indefinite distance.[6] On the other hand, in Armco wire and electrolytic wire, under precisely similar conditions, complete transmission returns within one second or less.[2] From evidence not yet published (having reference to the rates of recovery in different strengths of acid) it seems probable that the film formed on steel wire is at first relatively thick, and that recovery is the result of its progressive thinning by the solvent action of the acid, a certain limiting thickness (probably monomolecular) being eventually reached, corresponding to the final state of maximum transmissivity. In pure iron wire the passivating film is from the first thin; hence recovery is rapid. The influences of temperature and concentration of acid on the rate of recovery in Armco wire are similar in their general features

[5] Theoretically, a sufficient interruption of continuity is all that is required.

[6] Lillie, R. S., *J. Gen. Physiol.*, 1920, **3**, 107; 1925, **7**, 473.

to those found in steel wire, but the absolute rate of the process is much greater.

The rhythm is most readily demonstrated as follows. A short length of clean bright Armco wire (*c.g.*, 2 mm. thick, 1 to several cm. long) is placed in a flat-bottom vessel containing nitric acid of 60 to 80 v.% concentration.[7] Usually a rhythmical reaction starts at once; the metallic surface shows at regular intervals an alternation between a steel bright and a dull lustre; on closer examination it is seen that the effervescence is confined to the dark periods, while during the bright periods the metal is non-reactive (passive). The number of cycles per minute varies usually between 40 and 100 or more, increasing with the concentration of acid and the temperature. The relative duration of the passive phase in each cycle increases with the strength of acid; in the weaker acid the appearance is that of a bright flash passing at intervals over the dark effervescent surface of the metal. If the acid be left unstirred, the rhythm soon becomes irregular and passes over into a continuous effervescence; but with stirring a regular rhythm may last indefinitely, *i.e.*, until most of the wire is dissolved.[8]

Experiment shows that under these conditions the presence of the continually active or controlling region depends on the contact of the metal with the glass. At some local area of contact the diffusion of reaction products and acid is retarded and the acid soon becomes too weak to repassivate the iron; the reaction then becomes continuous, *i.e.*, a permanently active or anodal area is established. A wire freely suspended in acid by thin glass filaments is non-rhythmical; when activated it shows a single reaction and immediately becomes again passive and remains so. Rhythm, however, can readily be induced by locally interfering with diffusion or otherwise maintaining a permanent local activity.[9] The method finally adopted was to insert one end of the suspended wire into the interior of a narrow glass tube for a few millimeters. When such a wire is activated a few times (by touching

[7] Volumes of HNO_3, C. P., Sp. Gr. 1.42, in 100 volumes of solution.

[8] For further details *cf.* Lillie, R. S., *loc. cit.*[2]

[9] *E.g.*, continual contact of copper, zinc or other metal, anodal in relation to passive iron, will induce rhythm; but such rhythms are irregular because of the difficulty of maintaining constant conditions.

with zinc) continuous effervescence soon appears in the enclosed region, and waves of activity then pass at regular intervals from this region along the whole wire. When the acid is stirred sufficiently to render the local conditions stationary, the rhythm preserves its regularity; otherwise it fluctuates and tends to accelerate. Withdrawing the wire from the tube or otherwise arresting activity in the the pace-making area (*e.g.*, by contact with platinum) at once arrests the rhythm, and the wire becomes permanently passive, Conversely, increasing the active area, as by inserting the wire farther into the tube, accelerates the rhythm.

The manner in which the rhythm is influenced by the extent of the controlling active area, temperature, concentration of acid, electrical polarization of the wire by an external current, and length of wire have been briefly described in the two papers already cited.[9a] In the present paper the electromotor variations accompanying the rhythm will be described in some detail and their chief resemblances to the biological electromotor rhythms indicated. The variations of reaction velocity in the pulsating wire are an index of parallel variations in the passivating surface film, a regular rhythm implying alternate formation and breakdown of the film. The electrical variation is the most convenient and sensitive indicator of these changes in the film. Since there is evidence that variations in the semi-permeable protoplasmic surface films, affecting their permeability and electromotor properties, determine the bioelectric variations (membrane theory of Ostwald, Bernstein and their successors), the parallels between the electromotor variations in the two types of system are of interest as indicating the nature of the fundamental conditions determining activity in the irritable living system. In both cases the variations in chemical and electromotor activity appear to be primarily dependent on variations in the structure and composition of interfacial films.

The wire (Armco, *ca.* 2 mm. in diameter) was bent so that a straight portion of its length, *e.g.* 6 cm. long, could be immersed horizontally

[9a] Another form of rhythmical action is seen in "circuit transmission" in which a single "trapped" activation wave travels continually round and round a circular wire immersed in 80 v.% HNO_3. Each region of such a wire shows a regular rhythm determined by the rate of travel of the wave. For a description of this phenomenon *cf.* R. S. Lillie, *Science*, 1929, 69, 305.

in the acid, which was contained in a vessel 10 cm. in diameter provided with an outflow tube inserted into its side 1.5 cm. above the bottom. The other end of the wire was attached to a key (Harvard cross circuit type) from which connection was made through a rheostat to the string galvanometer. The other electrode was a strip of platinum foil placed parallel to the wire at 2 to 3 cm. distance. A short portion of the wire, including the bend where it passed from the acid into the air, was coated with paraffin (to prevent irregular action which otherwise occurs at this region), leaving a length of *ca.* 5 cm. exposed to the acid. Opposite the free end of the wire was placed a glass tube, of calibre slightly greater than the diameter of the wire, into which the latter could be inserted for a short distance. In these experiments the inserted length was adjusted so as to secure the slowest rhythm that was stable under the conditions. The platinum electrode and the wire were connected with the terminals of a tube rheostat of low resistance (29 ohms), and wires led from the sliding contact and one terminal to the string galvanometer, a small instrument of the permanent magnet type. The tension of the string and the distance between slide and terminal of the rheostat were kept constant throughout the experiments. The speed of the recording surface was also constant at 0.8 cm. per second. The acid was led into the vessel by a siphon with its outlet near the insertion of the wire into the glass tube. The slow flow of acid provided the necessary stirring.

The records contained in the present paper were made at room temperature (20° to 22°). Under constant external conditions, with rhythms satisfactorily uniform, different wires show some variation in the rate of rhythm and in the form of the curve shown in each single cycle, but the essential features of the phenomenon are remarkably constant. The rate of rhythm increases with increase in the concentration of acid between 60 and 80 v.%. On either side of this range regular rhythms are difficult to obtain; at 55 v.% the automatic return of passivity is uncertain and irregular, while at 80 v.% and higher the tendency to passivity predominates and the active period becomes extremely brief. The rhythms at 80 v.% are typically rapid, broken and irregular (Fig. 1). At 85 v.% no satisfactory rhythms could be obtained.

In a large number of observations the rhythms observed in the

different concentrations of acid at 20–22° fell nearly always within the following ranges:

Concentration (v. % HNO_3)	*Rate per minute*
60	30–40
65	45–60
70	60–80
75	85–100
80	120 or higher

An approach to a linear relation between rate and concentration seems indicated. The velocity and extent of the reaction in the pace-making area determine the rate at which iron ions pass into solution, and hence the intensity of the local activating current; and by the mass action law the reaction velocity should be proportional to the concentration of acid. But the area of the permanently active region cannot be controlled with any exactitude by the simple method used; it also varies as the reaction proceeds and the wire is dissolved away. There is also a rise of temperature in the enclosed column of acid, and this condition is probably largely responsible for the rapid acceleration of rhythm at the higher concentrations.

Of greater interest is the manner in which the form of the curve varies in the different solutions. Figs. 1 to 5 give typical examples of records obtained from short lengths of Armco wire under the conditions described. The concentrations of acid were respectively 80, 75, 70, 65 and 60 v.%. In these curves the upstroke corresponds to the change from passive to active, *i.e.*, the iron becomes more anodal. It is apparent that as the concentration falls the duration of the active phase increases; activity is also more readily maintained at a nearly constant level, *i.e.*, the tendency to passivity decreases. The plateau signifies a temporary maintenance of the active state; this feature of the curve becomes more pronounced with decrease in concentration. With still further decrease the active phase lengthens rapidly and becomes indefinitely prolonged at a concentration of *ca.* 55 v.%; *i.e.*, no spontaneous repassivation occurs in acid below this concentration. It is also noteworthy that the "dip" of the curve, representing the range of the variation of potential, is maximal at an intermediate concentration of 65 to 70 v.%. The period of steel-bright lustre is very brief in 60 v.% acid, as already mentioned, and the slightness of

the dip in this solution indicates that passivity is less completely attained than in stronger acid; this apparently signifies that the temporarily formed film covers a smaller portion of the surface.

In all cases the upstroke, signifying the breakdown of the passivating film, is rapid, reaching a maximum within a small fraction of a second. The process of repassivation, corresponding to the reformation of the film, then begins immediately, as indicated by the downward slope of the curve; this slope is steep and almost uniform in 80% acid and becomes more gradual as concentration decreases. The rather definite turning point or inflection in the downward slope, seen especially in the weaker acid, indicates that the passivation process undergoes rapid acceleration after reaching a certain critical stage. This stage may be taken as corresponding to the formation of a definite area of film-covered, *i.e.* cathodal, surface. Theoretical considerations indicate that as the cathodal area increases the total electric current between the active and the passive portions of the metallic surface also increases progressively up to a maximum;[10] and it is probable

[10] This current, other conditions being equal, would be maximal with equal areas of anode and cathode, since in general the strength of current in a battery system is directly proportional to the area of each electrode, *i.e.*, to the product of anodal and cathodal areas. If the area of the whole surface of the wire is unity, and if we regard anodal and cathodal areas as sharply defined, then if $\frac{1}{n}$ is the cathodal area, $\frac{n-1}{n}$ is the anodal area. The product of the two, $\frac{n-1}{n^2}$, is maximal when $n = 2$, *i.e.*, when the wire is half covered with the film.

It is probable, however, that the *density* (*i.e.*, intensity ÷ electrode area) of the local current traversing the surface of the wire, rather than its total intensity, is the chief factor to be considered, since the density determines the rate of electrochemical reduction or oxidation in each unit area of surface and hence the rate of the activating or passivating influence at the region concerned.

Designating the electrode areas as above, we see that over the cathodal surface the average current density is $\frac{n-1}{n^2} \div \frac{1}{n} = \frac{n-1}{n}$; over the anodal surface it is $\frac{n-1}{n^2} \div \frac{n-1}{n} = \frac{1}{n}$. That is, the average current density at any time over either electrode surface is directly proportional to the area of the other electrode surface. This shows, *e.g.*, that the density of the current at the cathodal (*i.e.* passive) surface increases linearly as the anodal surface increases, and *vice versa*. Activation

that the increase in the rate of passivation (*i.e.* of film formation over the previously active areas) corresponds to a critical increase in the intensity or density of this current. Anodal oxidation, recognized as a chief general means of inducing passivity in metals, is undoubtedly a main factor in the reformation of the passivating film.[11]

In addition to these larger rhythmical variations of potential slight irregular fluctuations occur under a variety of conditions. In curves with well defined plateau (*e.g.* Figs. 4 and 5) a slight notch or dip is often seen just before the final rapid descent which accompanies repassivation. When the active phase is greatly prolonged, as in acid between 55 and 60 v.% concentration, an irregular rhythmical fluctuation often lasts throughout the entire active period. Similar variations frequently occur during the passive phase, especially when the "pause" is prolonged. Figs. 6 and 7 illustrate this condition. In the case illustrated by Fig. 6 the wire spontaneously ceased activity after a series of regular pulsations in 65 v.% acid (at 22°), apparently as a result of the dissolving away of the free end inside the glass tube. The last few beats show retardation with an accompanying increase in the range of variation; the potential then falls progressively, with irregular fluctuations indicating an oscillation between activating and passivating influences, to a level corresponding to complete passivity. In general, when the interval between successive cycles of a rhythmical series is prolonged, the variation of potential is increased;

requires more than a certain critical density of current; hence slightly scraping a passive wire in HNO_3 with glass may fail to activate the wire as a whole—*i.e.*, fail to cause a transmitted effect—while a larger scrape activates.[6] Correspondingly, any local active area in a passive wire will spread, with an automatic acceleration (*i.e.* explosively), as soon as the area becomes large enough; similarly (although the conditions differ in detail) with a passive area. The steepness in both the ascent and descent of the curves may thus be understood. The irregular oscillations about a midway position shown in Figs. 6 and 7 are also probably an expression of the mutual influence exerted by local anodal and cathodal areas. The existence of such an influence between the active and passive areas of the same wire was early noted by Schönbein (Philos. Mag., 1836, Vol. 9).

[11] This influence is seen (*e.g.*) in the retardation and arrest of the rhythm of a pulsating wire when it is made the anode of an external circuit.[2] The relative duration of the passive phase is at first lengthened, and with sufficient polarizing current the wire soon comes to rest in the passive state.

the form of the curve also undergoes a change which is illustrated in Figs. 6 and 7.

It will be seen from Fig. 6 that the whole variation of potential between activity and complete passivity in 60 v.% acid has approximately twice the range of that shown in each single cycle. The potential difference between completely active and completely passive wires, in 60 to 70 v.% HNO_3, as shown when the two are connected through a voltmeter, is of the order of 0.7 volt. During the rhythmical series the variation is *ca.* 0.35 volt, *i.e.*, the wire is reactivated before passivation has reached the complete or equilibrium stage. That this is the case is also shown by comparison with Fig. 8 which gives records of single variations resulting from successive brief contacts of zinc with a non-rhythmical wire in 65 v.% acid at intervals of a few seconds (22°). The variations are seen to be uniform in character and duration, but of approximately twice the range characteristic of each cycle of the rhythmical series.

In the comparison with living tissues the more general conditions are of chief interest and may be here briefly reviewed. The known relations between stimulation and variation of permeability indicate that alternate breakdown and reformation of interfacial films are the controlling factors in the living as well as in the non-living system, stimulation corresponding to breakdown of the protoplasmic surface film and recovery to its reformation. In both systems these processes are under electrical control. It is also to be noted that in general the recovery in living tissues is more directly associated with consumption of oxygen than is the stimulation; this would indicate that the normal semi-permeable properties of the film in the resting or recovered cell are determined by some oxidation product or products, or by some by-product of an oxidative reaction. Alternate oxidation and reduction are the essential features of the chemical reaction cycle in the passive iron system; and such general physiological facts as polar stimulation and electrotonus indicate that in this respect also the general conditions in irritable protoplasmic systems are similar. The complete dissimilarity in the chemical details of the controlling surface reactions in the two cases is in no sense incompatible with an identity in the fundamental physical conditions of the reactions.

The precise form of the electromotor curve in the rhythmical iron system shows considerable variation, as the foregoing examples illustrate. Similarly the form of the electrocardiogram varies widely in different animals;[12] these variations are to be referred in part to special anatomical peculiarities, as well as to differences in the time-relations and other features of the characteristic reaction cycles. In simple invertebrate hearts curves resembling closely those of the iron wires occur frequently. A rapid upstroke, sloping plateau and rapid downstroke are seen, for example, in the hearts of mollusca (Aplysia[13]) and Crustaceans (Maja,[13] Homarus[14]). Oscillations in the plateau are a regular feature of the hearts of Maja and Limulus.[13] In the vertebrate heart, with its several chambers and differentiation of conducting and contractile tissues, such resemblances are less evident, and variations in the position of the leading-off electrodes affect greatly the form of the curve. In many cases, however, the electromotor variation of a single chamber (*e.g.* ventricle) conforms to this type of curve.[15]

SUMMARY

1. The electromotor variations of pure iron wires, arranged to react rhythmically with nitric acid, are recorded and described.

2. Resemblances between these variations and those of rhythmically reacting living tissues (especially the heart) are pointed out and discussed.

EXPLANATION OF PLATES

PLATE 1

Figs. 1–5. Tracings from wires in HNO_3 of the concentrations 80, 75, 70, 65 and 60 v.%. Temperature 22°. The respective rates per minute are 140–150, 96–98, 60, 46 and 35.

---

[12] For a review *cf.* the article by W. Einthoven, Handbuch der normalen und pathologischen Physiologie, 1928, 8, 785.

[13] Hoffmann, P., *Archiv f. Anat. u. Physiol., Physiol. Abth.*, 1911, 135; *cf.* Fig. 14, Taf. X. *Cf.* also the tracings from the heart of Helix in the article of C. Lovatt Evans, *Zeitschr. Biol.*, 1912, 59, 397, Fig. 3 and Plate VI, *A*, *B*, *D*.

[14] Hogben, L., *Quart. J. Exper. Physiol.*, 1925, 15, 264; *cf.* Figs. 21, 22.

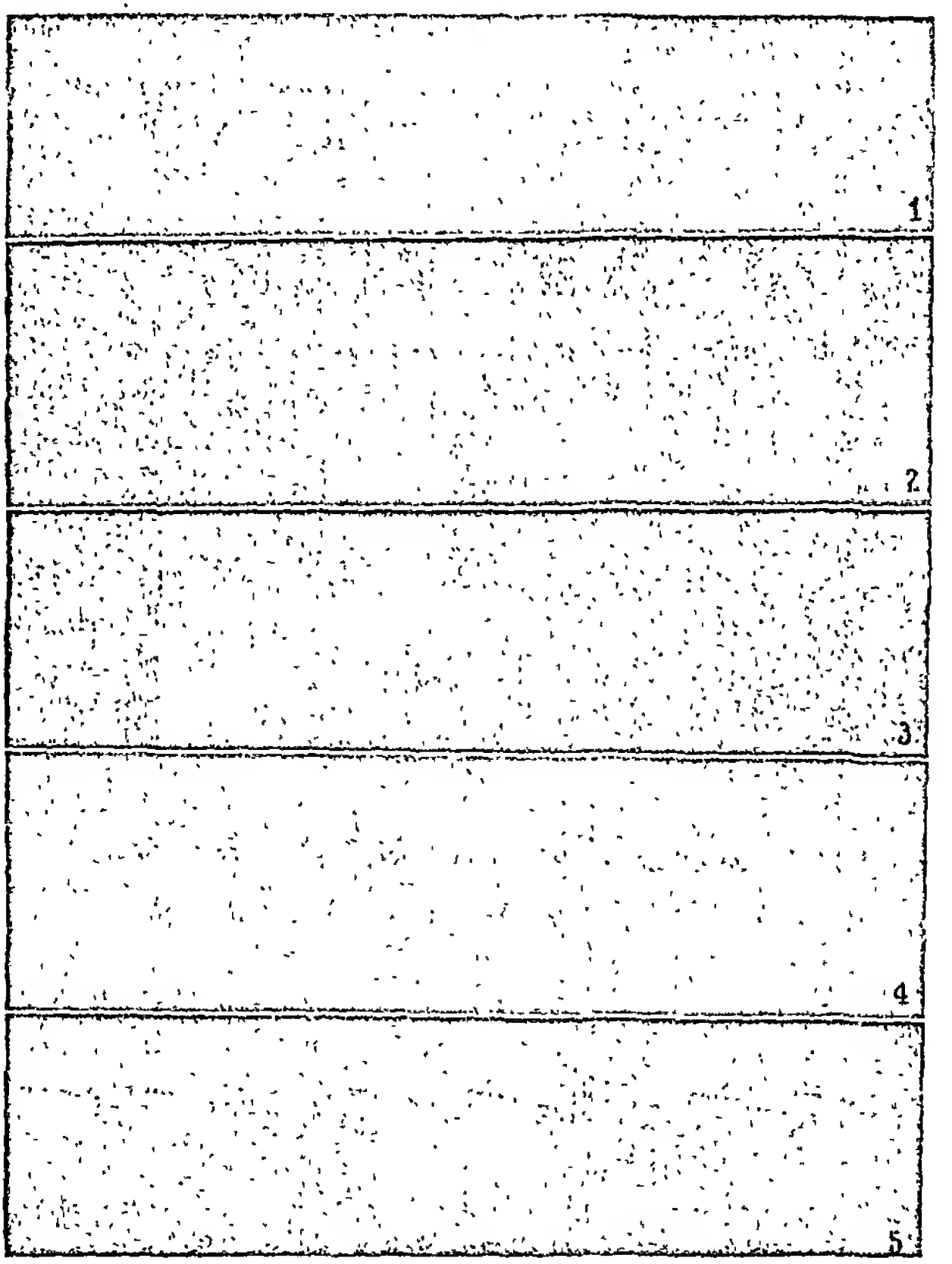
[15] *Cf.* the tracings from the frog's heart in Einthoven's article,[12] pp. 813, 849.

PLATE 2

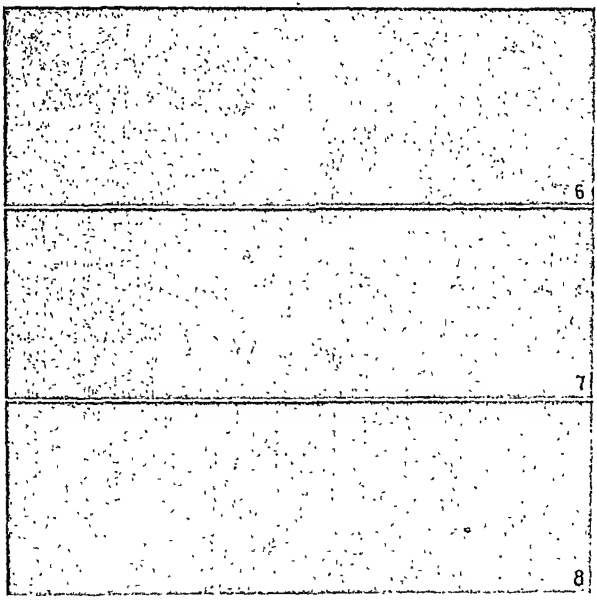
Fig. 6. Tracing from wire in 65 v.% HNO_3 showing relapse from rhythmical to permanently passive state at the end of a series of pulsations. Note rapid oscillations of potential during the relapse. Temperature 22°–23°.

Fig. 7. Another part of the previous tracing showing prolonged pause with oscillations of potential between pulsations.

Fig. 8. Variations resulting from successive contacts of a non-rhythmical wire with zinc. HNO_3 65 v.%. Temperature 22°.



(Lillie: Resemblances between electromotor variations)



(Lillie: Resemblances between electromotor variations)

A PRELIMINARY STUDY OF THE REDUCING INTENSITY OF LUMINOUS BACTERIA

By E. NEWTON HARVEY

(From the Physiological Laboratory, Princeton University, Princeton)

(Accepted for publication, May 22, 1929)

The studies of Clark[1] and his co-workers, and of Conant,[2] have called attention to the significance of oxidation-reduction potential as a measure of the reduction intensity of a chemical system, comparable to acidity. The investigations of Gillespie,[3] Cannan, Cohen, and Clark,[4] Coulter[4a] and Dubos[5] have shown the significance of the concept for cell suspensions, while the work of the Needhams,[6] Rapkine and Wurmser,[7] and Cohen, Chambers, and Reznikoff[8] has been concerned with the interior of the cell.

Briefly, it has been observed that different cells or cell suspension in absence of oxygen, possess a reduction intensity constant at a certain level, depending on the type of cell, which can be determined by ability to reduce some but not others of a series of oxidation-reduction (redox) indicators of different reduction intensity. When gold or platinum electrodes are placed in such suspensions the final electrical potentials obtained correspond to those expected from the redox indicator series. Some anaerobes may carry the potential to the overvoltage side of the hydrogen electrode and actually form hydro-

[1] Clark, W. M., and collaborators, Collected Studies, 1923-26, Bull. No. 151 Hygienic Lab. U. S. Pub. Health Service, *Chem. Rev.*, 2, 127, 1925.

[2] Conant, J. B., *Chem. Rev.*, 3, 1, 1926.

[3] Gillespie, L. J., *Soil Science*, 9, 199, 1920.

[4] Cannan, Cohen and Clark, Supp. No. 55 to Pub. Health Reports, 1926.

[4a] Coulter, C. B., *J. Gen. Physiol.*, 12, 139, 1928; Coulter and Isaacs, *J. Exp. Med.*, 49, 711, 1929.

[5] Dubos, R., *J. Exp. Med.*, 49, 507, 559, 575, 1929.

[6] Needham and Needham, *Proc. Roy Soc.*, 98, 259; 99, 383, 1926; *Protoplasma*, 1, 255, 1926.

[7] Rapkine and Wurmser, *Proc. Roy Soc.*, 102, 127, 1926.

[8] Cohen, Chambers and Reznikoff, *J. Gen. Physiol.*, 11, 585, 1928.

gen. In aerated suspensions the observed potential is naturally much further toward the oxidizing side and correspondingly redox indicators cannot be reduced that reduce easily under anaerobic conditions, but even so both cells and cell suspensions possess far greater reducing intensity than would correspond to the air electrode and indicators are held reduced that oxidize readily in air. Both suspensions and cells are rather poorly poised, but new reducing material seems to be slowly mobilized from some large reserve. Perhaps it should be emphasized that the above statements apply when the redox indicator is injected into a cell as well as when cell suspensions in the redox indicator solutions are used.

Luminous bacteria offer special advantages for oxidation-reduction studies, since the luminescence is an indication that oxidative reactions connected with light production are proceeding within the cell. In the crustacean, *Cypridina*, luminescence is connected with the oxidation of luciferin to oxyluciferin in presence of luciferase. *Cypridina* luciferin can only be oxidized to oxyluciferin rapidly by such systems as quinhydrone (not by o-chloro-indophenol) while oxyluciferin can only be rapidly reduced by such systems as anthraquinone 2, 6 di Na sulphonate. Between these systems luciferin is not oxidized by an oxidant and oxyluciferin not reduced by a reductant, so that we are not dealing with a truly reversible equilibrium and I have spoken of the apparent oxidation and apparent reduction potential of luciferin-oxyluciferin in Conant's[2] sense (Harvey[9]).

Luciferin and luciferase have never been demonstrated surely in luminous bacteria and the luminescence of these forms seems to be connected closely with other oxidative processes in the bacteria, since it is dimmed by dilute KCN which has practically no effect on the luminescence intensity of *Cypridina*. It should be pointed out, however, that KCN has a far greater effect in suppressing the respiratory oxidations than in decreasing bacterial luminescence (Harvey[10]) and Shoup[11] has recently shown that the oxygen consumption can be reduced to one-half the normal value by low concentrations of oxygen

[9] Harvey, E. N., *J. Gen. Physiol.*, 10, 385, 1927.
[10] Harvey, E. N., *J. Gen. Physiol.*, 8, 89, 1925.
[11] Shoup, C. S., unpublished observations.

before the luminescence intensity is affected. The respiratory and luminescence oxidations are not equally affected.

Nevertheless there are certain phenomena connected with the luminescence of bacteria as affected by the redox indicators, that suggest rapid oxidation of luciferin, although the interpretation of effects on luminescence are difficult. These will be considered below.

These oxidation-reduction studies were carried out on a suspension of the bacterium, a rod-shaped form isolated from fish and identified as *Bacillus fischeri* by Dr. S. E. Hill,[12] in M/4 phosphate buffer of $P_H = 7.6$, in which respiration and luminescence continue for some hours. If allowed to stand they use up the oxygen, when their luminescence disappears, and produce CO_2 in corresponding amount, but an amount which is too small to change the P_H of the highly buffered medium. The oxidized indicator[13] solutions were made up fresh and placed in tubes in small amount and sufficient thoroughly aerated concentrated bacterial suspension added to bring the indicator concentration to approximately M/100,000 to M/10,000. No doubt the proper method of comparing redox indicators is in equimolecular concentration but some indicators are so highly colored as compared with others in equimolecular concentration that the color obscures the luminescence.[14] I have found it better in practice to add enough indicator to give just observable color that does not absorb much of the light from the baceria. It must be borne in mind that too much indicator added to bacterial suspension will overbalance the reducing capacity of the bacteria and no reduction of the indicator will occur even though plenty of time is allowed. The luminescence will also be permanently quenched by higher concentrations of indicators.

The mixture of indicator and bacterial suspension then stood undisturbed and the effect on luminescence and on color change in the indicator noted, especially the relation between reduction of the indicator and disappearance of luminescence. Part of the time for

[12] Hill, S. E., Biol. Bull., 55, 143, 1928.

[13] Most of the indicators were obtained from the La Motte Chemical Products Company. I am deeply indebted to Dr. Barnett Cohen for Nos. 5, 6, 13 and 17, and to Dr. J. B. Conant for Nos. 9, 10, 14, 15, 21, and 22.

[14] Quinone has a far greater effect in dimming a suspension of luminous bacteria than o-Cl-indophenol in the same molecular concentration.

disappearance of color is that necessary for the bacteria to use up the oxygen in solution and part, the time required to reduce the dye. Of course luminescence always disappears when dissolved oxygen has been utilized by the respiration.

Observations were also made on the intensity of luminescence and the reoxidation of the reduced dye by air, when the suspension is again thoroughly aerated. Table I gives these general relations for a series of reversible indicator and oxidation-reduction systems. Some are known to be relatively harmless for sea urchin eggs[8] (Column 7) and present no obvious toxic effects on luminous bacteria (Column 8). Others are definitely toxic and for this reason it is somewhat difficult to analyze their effects.

Considering first Column 5, it is generally true that reduction is easiest the higher the indicators stand in the series. This is shown by the time for reduction, which in general lengthens as we proceed downward in the series and especially by the fact that some of the dyes can be reduced before the luminescence disappears or dims, others reduce simultaneously with the dimming of luminescence, and others only after all trace of luminescence has disappeared. The indigo mono-sulphonate cannot be reduced at all. These relations are indicated by the letters a, b, c, which come in order from above down.

Shoup's studies[11] in this laboratory have indicated that the luminescence of an emulsion of bacteria just begins to dim at about 0.26 per cent oxygen (2.6×10^{-3} atmosphere). At oxygen pressures above this value the luminescence intensity remains the same. A just perceptible luminescence can be detected with an oxygen pressure of 0.0007 per cent (7×10^{-6} atmosphere) (Harvey and Morrison[15]) but this value is only to be regarded as an order of magnitude, since it depends on many variable factors such as dark adaptation of the eye, thickness and concentration of bacterial emulsion, etc. The reduction of some indophenols while the luminescence is still bright indicates that there must be present in the emulsion 0.26 per cent oxygen, an amount far above the theoretical pressure of oxygen that would be in equilibrium with an electrode in contact with reduced indophenol. The observation simply shows that the reduction of

[15] Harvey and Morrison, *J. Gen. Physiol.*, 6, 13, 1923.

TABLE I

	Molecular weight	E_0 at $P_H = 7$	R_H	R_O	Color change	Luminescence	Toxicity to *Amoeba dubia*	Toxicity to luminous bacteria
O_2 electrode		+0.810	41.0	0				
Air electrode		+0.800	40.7					
1. $K_4Fe(CN)_6$	329.2	+0.430	28.4			2		N
2. Quinone	108.0	+0.278				1		T
3. o-Cl indophenol	255.6	+0.233	21.8	38.5	ax	1′	N	N
4. Phenol indophenol	221.0	+0.227	21.6		ax	1′	N	N
5. Bindschedler's green, base	256.0	+0.224	21.5		ay	1	T	T
6. [illegible]						1	N	S (?)
7.						1′	N	N
8. 2,6 di Cl 3 methyl indophenol	305.0	+0.181	20.1		ab xy	1	S	S
9. p-xyloquinone	136.0	+0.170				1		T
10. 1,2 napthoquinone	158.0	+0.127				1		T
11. 1 napthol 2 SO_4 indophenol	373.0	+0.123	18.1		ab y	2	S	N
12. 1 napthol 2 SO_4 indo 2,6 di Cl phenol		+0.119	18.0		by	2	T	N
13. Toluylene blue chloride H_2O	308.0	+0.115	17.9		by	1	S	S (?)
14. 2 Cl 1, 4 napthoquinone	227.0	+0.080				1		T
15. 1,4 napthoquinone	158.0	+0.070				1		T
16. Methylene blue Cl $5H_2O$	410.0	+0.011	14.4		by	2	S	N
17. K_4 indigotetrasulphonate	734.0	−0.046	12.5		by	2	T*	N
18. K_3 indigotrisulphonate		−0.081	11.3		by	2	T*	N
19. K_2 indigodisulphonate	498.0	−0.125	9.9		by	2	T	N
20. K indigosulphonate	380.0	−0.182	7.5	63.0	c	2		N
21. Na_2 anthraquinone 2,6 disulphonate		−0.192				2		N
22. Na anthraquinone 2 sulphonate		−0.233				2		N
H_2 electrode		−0.421	0	82.0				

a. Color fades before luminescence disappears. Reduction.
b. Color fades after luminescence disappears. Reduction.
c. Color does not disappear. No reduction.
x. Color disappears while air bubbled through; no return on shaking with air.
y. Color remains while air bubbled through; returns on shaking with air.
1. Luminescence progressively dims.
1′. Luminescence dims at first but returns again.
2. Luminescence not dimmed until oxygen used up.

Toxicity for *Amoeba* after Cohen, Chambers and Reznikoff.[3] Toxicity for bacteria judged by progressive dimming and failure of light to return on shaking thoroughly with air. T, toxic; T*, oxidant toxic, reductant non-toxic; S, slightly toxic; N, non-toxic.

indophenol in the bacterial suspension is no indication that the oxygen has been reduced to a very low figure and it is quite possible that in suspensions of bacteria containing reduced methylene blue, even though no luminescence is visible, the concentration of oxygen is not so low that the "last molecule" has disappeared, *i.e.*, the oxygen pressure may not necessarily be that theoretically in equilibrium with an electrode in contact with reduced methylene blue. The R_o values given in the table are $\log \frac{1}{P_o}$ values where P_o = oxygen pressure in atmospheres. The oxygen pressure in the case of o-indophenol would be theoretically $10^{-38.5}$ atmosphere, whereas the bacteria are still luminescing brightly.

From the data in the table we can put the reduction potential of the bacterial suspension in air in the R_H region 18 to 20 whereas the reduction potential in absence of oxygen is in the region $R_H = 8$ to 10. We might predict that potentiometric observation with the gold electrode in nitrogen should give an E_H value around -0.20 volts or a potential of -0.446 against the saturated KCl calomel electrode. One of my students, Mr. Emerson Holcomb, is now engaged in a study of these potentials and a quantitative investigation of the reduction of these systems, penetration, the place where reduction occurs, whether inside or outside the cell, etc.

The reduction of a number of other dyes whose position in the redox scale is not accurately known was also tested, namely: trypan red, alizarin blue S, Na carminate, bismark brown, neutral red, trypan blue, anilin green, brilliant cresyl blue, bordeaux red, toluidine blue, janus blue, methyl violet B, thionin, isamine blue, methyl green, diazin green, rhodamine, vital red HR, neutral fuchsine, nile blue and safranin. Of these, only brilliant cresyl blue, toluidine blue, and thionin were reduced.

These luminous bacteria are obligatory aerobes (Shoup[16]) and their behavior toward indicators is what we should expect from our general knowledge of the behavior of aerobic cells and suspension toward the redox indicator series.[4] The luminescence does not endow them with any unusual behavior.

[16] Shoup, C. S., *Proc. Soc. Exp. Biol. Med.*, 25, 570, 1928.

When effects of the redox series on luminescence are analyzed we can make the general statement that the more oxidizing members readily dim the luminescence while the more reducing members (in the oxidant form) do not, apart from the absorption of light which results from their color. One might interpret this as meaning that their redox potential is greater than that of luciferin and consequently they oxidize most of the luciferin, leaving none or little for luminescence. Such an interpretation is attractive and from what we know of the behavior of *Cypridina* luciferin quite applicable to quinone, but in view of the fact that 1.4 napthoquinone, which stands low in the series, also causes rapid dimming as well as other napthoquinones and xyloquinone, we cannot regard this explanation as unequivocal. It may merely be that the quinones in general are toxic, for the luminescence of bacteria can be dimmed by anesthetics and other substances which have no oxidizing action. However, the anthraquinone sulphonates (in oxidized form) do not affect the luminescence and also stand well down in the series.

One might expect that K ferricyanide, a strong oxidizing substance and hence one which should immediately oxidize luciferin, would dim the light and yet there is no effect with either K or NH_4 ferricyanide, even in M/1000 concentration. I interpret the lack of effect to mean that these salts cannot penetrate the bacterial cell. $KMnO_4$ dims somewhat in M/5000 concentration, whereas quinone prevents luminescence entirely at far greater dilutions than this.

The chief interest lies in systems of which o-Cl-indophenol is a good example, which, in weak concentrations, cause an initial dimming of the luminescence which soon returns to its original brightness. This recovery of the luminescence intensity occurs as the color of the indicator fades but is not entirely connected with absorption of light, as dimming occurs in concentrations so weak as to have practically no color. I interpret it to mean a rapid oxidation of luciferin by the indophenol so that no luciferin is left in the cell to produce light. As the indophenols are reduced more luciferin can be produced and the luminescence returns. The dyes are reduced while the luminescence is bright, showing that oxygen is present, and in fact the dyes can be reduced while air is bubbled through the suspensions as has been observed with many indophenols.[4] Addition of ferricyanide can

immediate return of the dye color by reoxidation and a second dimming of luminescence. So long as we hold the indophenol in the oxidized form the luminescence is dimmed.

The indicator dyes having an R_H of 18.1 or less, with the possible exception of toluylene blue, do not seem to affect the luminescence in small amount apart from the absorption of light due to their color, and it is especially interesting to note that the colorless anthraquinones do not interfere with the luminescence, and should not from their position in the redox series.

SUMMARY

The effect of a series of redox indicators and systems has been tested with a suspension of luminous bacteria (*B. fischeri*) in M/4 phosphate buffer of $P_H = 7.6$.

The indicators behave as expected from their position in the redox series, the most positive being reduced rapidly even in presence of air and before luminescence of the bacteria disappears, those of intermediate position at the time luminescence disappears, and the more negative only long after the luminescence had ceased, due to utilization of oxygen by the bacterial respiration. Indigo monosulphonate was the only indicator not reduced on long standing of a bacterial suspension. The aerobic redox potential may be placed at an $R_H = 18–20$ and the anaerobic potential at an $R_H = 8–10$.

Ferricyanides do not affect luminescence and behave as if they could not penetrate the bacterial cell. Quinone and the napthoquinones cause progressive dimming of luminescence in any concentration which affects the light but it cannot be definitely stated that this is due to rapid oxidation of luciferin although it seems likely in the case of quinone. Some indophenols dim the luminescence at first, followed by return of brightness, which is interpreted to mean rapid oxidation of luciferin while the indophenol is unreduced, more luciferin production after reduction of indophenol. The more negative redox systems do not affect the luminescence. Investigation of indicator reduction and luminescence is being continued.

UNEQUAL DISTRIBUTION OF IONS IN A COLLODION CELL

By JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, May 25, 1929)

Collander (1) found that the permeability of "dry" collodion membranes was essentially the same as that of living cells in that they allowed the passage of weak acids, bases, etc., but not of salts nor strong acids. Living cells possess another striking characteristic in that they are able to maintain a constant difference in concentration of solutes between the interior of the cell and the surrounding solution. This behavior can be accounted for if it be assumed that the substance becomes changed after entering the cell to one which cannot pass through the membrane. The non-diffusible form will then concentrate inside the cell. The results obtained in the previous study (2) of dry collodion membranes predict the conditions necessary for this result.

Concentration of Iodide Ion

Dry collodion membranes are readily permeable to iodine but are impermeable to iodide ion or to thiosulphate. If, therefore, a saturated solution of iodine is separated from a solution of thiosulphate by such a membrane the iodine will diffuse through the membrane and become reduced to iodide which cannot diffuse out. The iodide will therefore continue to collect in the cell. This will continue until all the thiosulphate has been used up and the activity (vapor pressure) of the iodine becomes equal on the two sides of the membrane.

The result of an experiment set up in this way is shown in Fig. 1. The membranes were made as previously described and were suspended in 500 cc. of a saturated solution of iodine in water with excess solid iodine present. The concentration of iodine outside remained constant. The concentration of iodide inside increases slowly, and until about the 50th day no iodide was found outside showing that the

brane is impermeable to both thiosulphate and iodide. At this time the membranes became yellow and crinkled and the concentration of iodide inside suddenly increased, while at the same time iodide began to appear in the outside solution. The membranes had evidently lost their semipermeability and become like dead cells.

Concentration of Chloride Ions

It was found previously that mercury chloride passed through dry collodion membranes while all other chlorides tested could not pass through. A system, therefore, in which the mercury chloride was

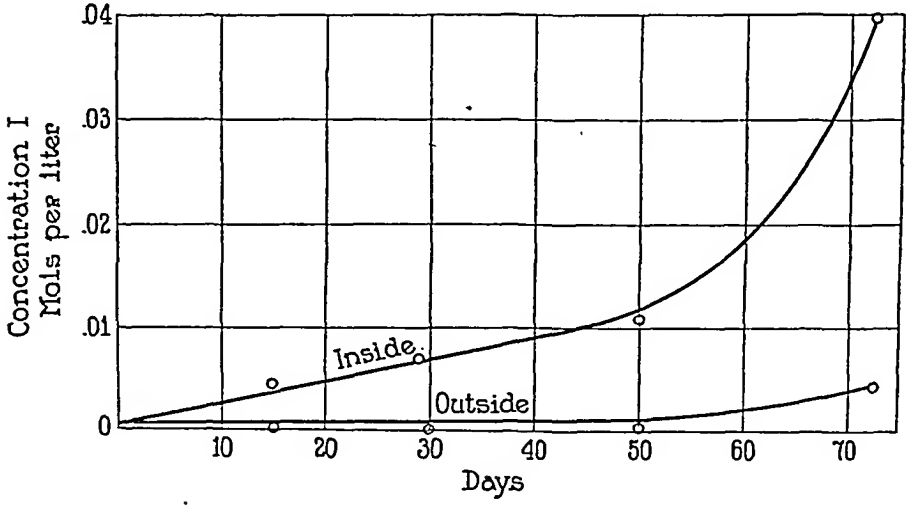


FIG. 1. Concentration of iodine in cell: $Na_2S_2O_3$ 0.10 M/I_2 — water.

changed to another chloride after entering the cell would concentrate chloride ions.

The results of such an experiment are shown in Fig. 2. The cell contained originally 0.011 M $HgCl_2$ and 0.05 M Na_2CrO_4 inside and 0.011 $HgCl_2$ and 0.05 Na_2SO_4 outside. On mixing the mercury was precipitated as $HgCrO_4$. The activity of the mercury chloride was therefore less inside than out and so more mercury chloride diffuses in and the chloride ion concentration increases as shown in the figure. This would continue until the product of the Hg and Cl ion activities inside and out were equal. Since the membrane is permeable for water it would also be necessary that the vapor pressure of the water

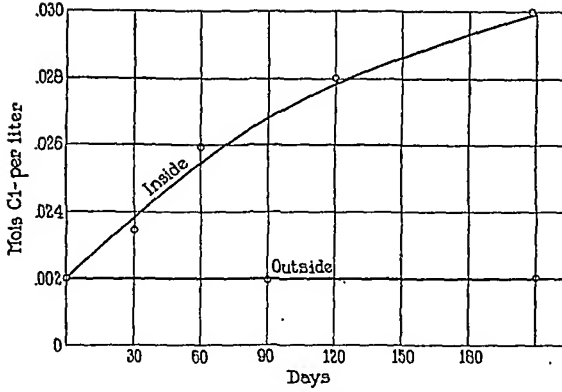


FIG. 2. Concentration of Cl^- ions in cell: 0.011 $HgCl_2$, 0.05 Na_2CrO_4/0.011 $HgCl_2$, 0.05 Na_2SO_4.

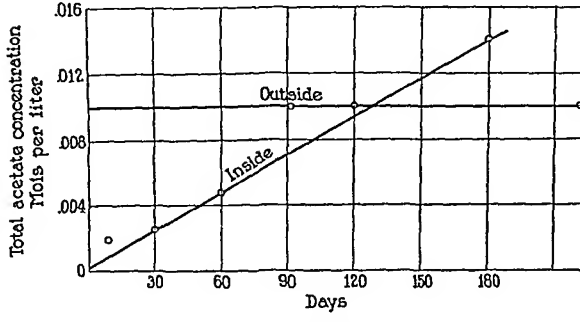


FIG. 3. Concentration of acetate ion in cell: 0.01 acetic acid/$CaCO_3$ — water.

be the same on both sides at equilibrium. In this case the condition is nearly fulfilled because of the sodium sulfate outside. The condition could be equally well fulfilled by exerting pressure on the inside solution.

Concentration of Acetate Ions

Acetic acid penetrates the membrane while acetate ion does not. If the acid is changed to an acetate after entering evidently acetate ion will become concentrated in the cell. This may be accomplished in a number of ways but the most convenient is to place solid calcium carbonate in the cell. The result of such an experiment in which a cell containing a suspension of calcium carbonate in water was placed in a solution of 0.01 M acetic acid is shown in Fig. 3. The concentration of acetate ion in the cell was determined by titrating a sample from pH 7 to pH 3.0 with 0.01 M HCl. The acetate ion concentration in the cell increases slowly and becomes greater than the total acetic acid concentration outside, and therefore many times greater than the acetate ion concentration outside. The process would continue presumably until the activity of the acetic acid (which is proportional to the product of the activities of the H times the acetate ion) was equal on the two sides. The vapor pressure of the water must also be equal at equilibrium due either to hydrostatic pressure on the inside or to the addition of some non-diffusible solute outside, or to proper adjustment of the acetic acid-calcium acetate concentrations.

This experiment is very similar to Osterhout's (3) results with H_2S and *Valonia* cells.

The preceding experiments show that a model may be made which will concentrate ions just as do living cells. It is, of course, unlikely that the same ions take part in the transfer in the case of the living cells but it seems possible that the general mechanism is the same. Since the permeability appears to be a property of the molecular species rather than the ion it is possible that the potassium or calcium salt of some organic acid is able to penetrate the cell membrane. Most organic acids are oxidized in the cell and if this occurred, the ion which entered with the organic ion could not escape and would be concentrated in the cell. There are evidently a number of other possibilities which would lead to the same result.

SUMMARY

The properties of dry collodion membranes previously described allow the prediction that cells of these membranes will concentrate solutes under certain conditions. Three such cases have been studied experimentally.

1. A membrane containing thiosulphate and immersed in a solution of iodine concentrates iodide ion.

2. A membrane containing sodium chromate and immersed in mercury chloride concentrates chloride ion.

3. A membrane containing calcium carbonate and immersed in acetic acid concentrates acetate ions.

BIBLIOGRAPHY

1. Collander, R., *Soc. Scientiarum Fennica Comment. Biol.*, 1928, 6, 2, 1.
2. Northrop, J. H., *J. Gen. Physiol.*, 1929, 12, 435.
3. Osterhout, W. J. V., *J. Gen. Physiol.*, 1927. 8, 131.

THE RESPIRATION OF LUMINOUS BACTERIA AND THE EFFECT OF OXYGEN TENSION UPON OXYGEN CONSUMPTION

By CHARLES S. SHOUP

(From the Physiological Laboratory, Princeton University, Princeton, and the Marine Biological Laboratory, Woods Hole)

(Accepted for publication, May 20, 1929)

INTRODUCTION

In the case of large organisms the rate of respiratory exchange will depend (1) on the amount of oxygen available for use, and (2) on adequate respiratory and circulatory mechanisms in the body. Amberson and his associates[3] in the case of several marine invertebrates, and S. Nomura[22] with the Holothurian *Caudina*, have shown that the rate of oxygen consumption is proportional to the oxygen tension of the medium. Pütter[26a] examined the oxygen consumption of several invertebrates at low pressures of oxygen and determined the limiting value for oxygen at which respiratory activity was equivalent to that in air. Very recently Hall[10] has shown the dependence of certain marine fishes upon the amount of available oxygen in the medium and the haemoglobin content of the blood for complete respiration. In all of these larger forms the rate of oxygen consumption is dependent upon oxygen concentration over a wide range of oxygen pressures.

In general, organisms such as protozoa or bacteria are small enough to allow complete diffusion of gases dissolved in the medium, and consume oxygen at a constant rate independently of the oxygen pressure until the oxygen concentration falls to a point allowing incomplete activity of the respiratory mechanism within the cell. Only then does the rate of oxygen consumption diminish, and in many forms this limiting value for adequate respiration will be very small indeed, as has been shown by various workers, (Warburg,[31a] Henze,[13] Lund,[19] Pütter,[26b] Harvey [11b]) whose experiments indicate that in unicellular

organisms oxygen consumption remains independent of oxygen concentration over a very wide range of oxygen pressure. Amberson[2] has shown that the oxygen uptake of Paramecium is practically constant from 200 to 50 mm. Hg oxygen, and the limiting value for adequate respiration in these organisms is not reached until the oxygen tension falls below 50 mm. At 11 mm. of oxygen the reduction in the rate of respiration is only 20 per cent of that at atmospheric pressure. Lund[19] has obtained results in agreement with those of Amberson on the same organism. Amberson also conducted experiments on the dividing egg of the sea-urchin and found that respiratory activity does not fall away from the rate at atmospheric pressure of oxygen until 80 mm. Hg is reached, and no marked decrease in the rate of respiration occurs until oxygen pressure has been reduced to 20 mm. Hg.

Estimations have been made of the rate of oxygen consumption with abundant oxygen by luminous bacteria when suspended in seawater, by methods involving the time for dimming (Harvey[11b]) and by a manometric method (Harvey[11c]). In the present investigations measurements have been made of actual values for oxygen consumption and carbon dioxide production when the amount of available oxygen is diminished, and also the rate of respiratory exchange before and following the dimming of luminesence of luminous bacteria due to lack of oxygen. The amount of oxygen necessary for the maximum luminesence is less than that required for maximum metabolism of the cell, and luminesence will still occur when only a very small amount of oxygen is present (Beijerinck,[5] Harvey[11d]). The actual amount of oxygen necessary to give just visible luminesence has been estimated by Harvey and Morrison[11e] to be of the small value 0.0053 mm. Hg (0.0007 per cent). At least 90 per cent of available oxygen is consumed when dimming of a suspension of luminous bacteria occurs.

In these experiments oxygen consumption by luminous bacteria has been followed by two methods: (1) Colorimetric, involving the use of haemocyanin as an indicator of the presence of dissolved oxygen in suspensions of bacteria, and (2) a manometric method allowing direct volumetric determinations to be made of the amount of oxygen consumed in given time.

Colorimetric Determinations of Oxygen Consumption

It is necessary to have a convenient indicator for the presence of oxygen if the rate at which oxygen is removed from a suspension of bacteria is to be observed. No colorimetric indicator of oxygen is available in which living cells may be placed excepting the blood-serum of certain crustaceans containing the copper-protein compound haemocyanin. The methods followed in these experiments are similar to those suggested by Osterhout[23] and Harvey[15] for the use of *Limulus* haemocyanin as an oxygen indicator. Luminous bacteria live perfectly well in the oxygenated *Limulus* serum, and as they consume oxygen from the serum in which they are suspended it passes from the dark blue of fully-oxygenated oxyhaemocyanin to the colorless reduced haemocyanin free of oxygen.

TABLE I

Color Standards

Tube No.	Oxygenated haemocyanin	
1	1 part to 9 parts sea-water	Each tube with 1 cc. bacterial suspension killed with toluol
2	2 parts " 8 " "	
3	3 " " 7 " "	
4	4 " " 6 " "	
5	5 " " 5 " "	
6	6 " " 4 " "	
7	7 " " 3 " "	
8	8 " " 2 " "	
9	9 " " 1 part sea-water	
10	10 " " 0 " "	

The color change of a standing tube of *Limulus* serum containing luminous bacteria was followed, and the actual time for the consumption of available oxygen was determined. The experimental tube containing living luminous bacteria in blood-serum was compared with a set of standards from dilutions of oxygenated *Limulus* haemocyanin to represent the shade of blue color present at definite percentages of oxyhaemocyanin. Corresponding oxygen tensions for each percentage of oxyhaemocyanin were taken from the haemocyanin-oxyhaemocyanin dissociation curve of Redfield, Coolidge, and Hurd.[27] Color standards were made as shown in Table I, using ten uniform test-tubes.

The killed bacterial suspension was added to give to the color standards the same degree of turbidity that is exhibited by an experimental tube prepared according to the same method.

A series of indicator tubes prepared as above served for comparison with a standing tube of *Limulus* oxyhaemocyanin containing 1 cc. of *living* luminous bacteria whose oxygen consumption was to be measured. The exact time was

taken whenever the experimental tube compared exactly in color with one of the standards. The blood-serum was thoroughly shaken with air until completely in equilibrium; the time was taken from the moment the luminous bacteria were added to the tube of serum. As the oxyhaemocyanin was reduced by the bacteria in the experimental tube confusing yellowish pigments appeared with the diminution of the blue color of the oxyhaemocyanin. A small amount of Orange G added to the color standards compensated for this color change in the experimental tube.

In Table II the corresponding oxygen tensions and volume percentages of oxygen for each standard tube are given. It will be seen that no color change occurs until the bacteria have reduced the oxygen concentration in the experimental tube to a value corresponding to 28.50 mm. Hg tension (3.75 per cent oxygen). This will correspond with standard Tube 8, one being scarcely able to distinguish a color change between standard No. 8 and standard No. 9, while no visible change from blue to less blue occurs above this value of oxygen. Oxyhaemocyanin is within 10 per cent of complete equilibrium with the air at this value of oxygen, and no distinction in color can be seen between 90 per cent and 100 per cent oxyhaemocyanin.

Considerable time was therefore required for the bacteria to consume enough oxygen to cause a reduction of the oxyhaemocyanin to a point resulting in color changes at which determinations could begin. From a beginning value of 23.50 mm. oxygen to a point equivalent to standard No. 1, or 3.75 mm. (0.493 per cent oxygen), it was possible to follow the changes in the color of the experimental tube and to obtain a curve for color values against time. The oxygen consumption curve obtained by this method is limited only by the extent of the color changes of haemocyanin when passing from the oxygenated to the reduced condition. Fig. 1 shows this relation plotted against time.

At the point of lowest possible concentration of oxygen estimated by the colorimetric method, the suspension of luminous bacteria in the serum was found to be still aglow. Dimming does not occur until after a complete reduction of the oxyhaemocyanin has been brought about. This may be determined by observing the experimental tube in the dark beside a control tube kept shaken and in equilibrium with the air. When the dimming of the experimental tube begins, it is easily noted by comparison with the brilliancy of glow in the control.

Table II gives complete data from a single experiment of oxygen consumption determination.

In this experiment no dimming of the suspension of luminous bacteria was detected until a total time of 16 minutes had elapsed, a point well beyond the time at which visible reduction of the oxyhaemocyanin had occurred. Hence, it is not possible to follow the rate of oxygen consumption beyond the point of dimming by this method. The character of the oxygen consumption curve beyond this point has been investigated by the manometric method to be described later in the present paper.

TABLE II

Experiment 2, July 11, 1927

Time in minutes and seconds	Standard tube No.	Per cent OxyHcy.	Oxygen tension in mm	Per cent oxygen in suspension	Log. of oxygen tensions
0.00	10	100	—	—	—
1.00	10	100 (?)	—	—	—
2.00	9	90	34.50	4.53	1.538
2.45	8	80	28.50	3.75	1.455
3.10	7	70	23.25	3.27	1.366
3.40	6	60	18.25	2.40	1.264
4.25	5	50	13.75	1.81	1.138
5.30	4	40	10.50	1.38	1.021
6.15	3	30	7.60	1.00	0.881
7.30	2	20	5.25	0.690	0.760
10.00	1	10	3.75	0.493	0.574

It may be noted in Fig. 1 that as oxygen consumption proceeds, equal parts of oxygen are consumed in equal times, independently of oxygen concentration (a "zero-order" reaction), down to a very low value of approximately 20 mm. Hg, before a marked reduction in the rate of oxygen consumption occurs. This is below a point corresponding with standard Tube 7, equivalent to 23.25 mm. oxygen, and standard Tube 6, which corresponds to 18.25 mm. oxygen. This is quite unlike the determinations made for larger animals where the oxygen consumption is proportional to the pressure over long periods of time and wide ranges of pressures. We cannot conclude that oxygen consumption of luminous bacteria is proportional to the oxygen

concentration except possibly at very low values of oxygen below the point of dimming. This is observed also in the complete curve obtained by manometric methods. Dimming of the suspension of bacteria occurs after the decrease in respiratory rate, indicating that the point of dimming is not the exact point at which oxygen concentration becomes just inadequate for maximum respiration of the cell.

The decrease in the rate of oxygen consumption must occur at a point where the active reactant oxygen is diminished to a value not

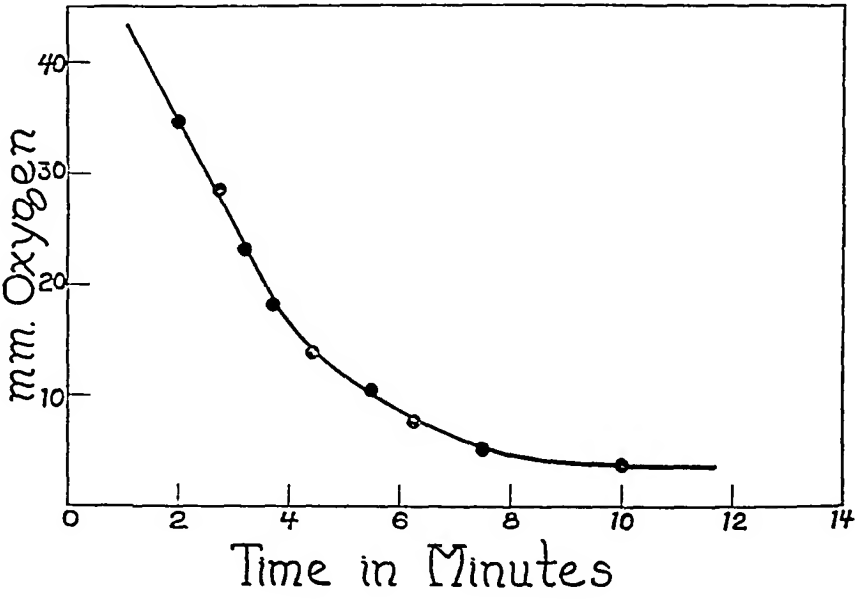


FIG. 1. Oxygen consumption of a suspension of luminous bacteria

quite sufficient to completely activate the respiratory mechanism of the cell, assuming that oxygen diffuses readily into these small organisms. This method for the determination of oxygen consumption is added to these studies to check later observations, and to call attention to the use of a colorimetric method for detection of the rate of oxygen consumption by small organisms. It should be borne in mind that there is no removal of the reaction product, carbon dioxide, but since *Limulus* blood is very well buffered, no inhibitory effects on the activity of the bacteria occurred, and the hydrogen-ion concentration of the serum did not change during the course of an experiment. The

greatest disadvantage of this method is that the range of the indicator is extremely limited and judgment of color values may often be inaccurate.

Colorimetric Determination of Carbon Dioxide Production by Luminous Bacteria

The amount of carbon dioxide produced in a standing tube containing a suspension of luminous bacteria in sea-water has also been determined by a colorimetric method. Haas[9] and Saunders[38] have previously used indicator dyes for the determination of carbon dioxide production by both marine and fresh-water animals. Henderson and Cohn[12] have published a table for the relation of the pH of sea-water to its carbon dioxide tension. By the use of indicator dyes it is possible to follow changes in the pH of a bacterial suspension in sea-water as the bacteria produce carbon dioxide and effect the bicarbonate buffer equilibrium.

Allowance was made for the salt-error introduced, from the tables of Kolthoff and Furman,[16] and careful check was made on the changing pH of the suspension in time. Curves plotted for the pH were expressed in terms of carbon dioxide tension according to the data of Henderson and Cohn. Suspensions of the bacteria were always shaken into complete equilibrium with the air and added to a test-tube containing the proper amount of the indicator (Phenol red), while a like amount of the suspension was added to a test-tube without dye to serve as a control for observation in the dark to establish time for dimming. At the end of each observation, when the experimental tube had reached the lowest pH measureable by the indicator, air was bubbled through the suspension to drive off the carbon dioxide produced and allow the suspension to return to near its original pH. This indicated that the change in pH was almost entirely due to the presence of the carbon dioxide produced by the bacteria. If the indicator did not return to within 0.15 of the original pH, the experiment was disregarded.

Although luminous bacteria are very active producers of acid, as Hill[14] has shown, a great acid production occurs only when they are suspended in a medium containing carbohydrates. Since these experiments were always conducted with bacteria suspended in a non-nutrient medium free of carbohydrates, the acid production was very slight, the only source of acid being from the carbohydrates of the cells themselves and dead bacteria in the suspensions. The experiments were conducted over relatively short time intervals, and acid production at no time became too great for reading the pH values or such as to obscure results. It will be seen from Fig. 2 that in a series of experiments on carbon dioxide production in samples from a single suspension of luminous bacteria, there is a slight

rise in the hydrogen-ion concentration of the suspension at all times. This is the only evidence of the slight amount of non-volatile acid that is produced by the bacteria as the suspension becomes older. However, only in old suspensions and suspensions made from old cultures were there sufficient quantities of acid to cause error to determinations. Consequently, all of the experiments were conducted with fresh and young, brilliantly-glowing cultures.

In Fig. 2 the rise in the amount of carbon dioxide produced in the suspension proceeds at a constant rate from equilibrium with the air

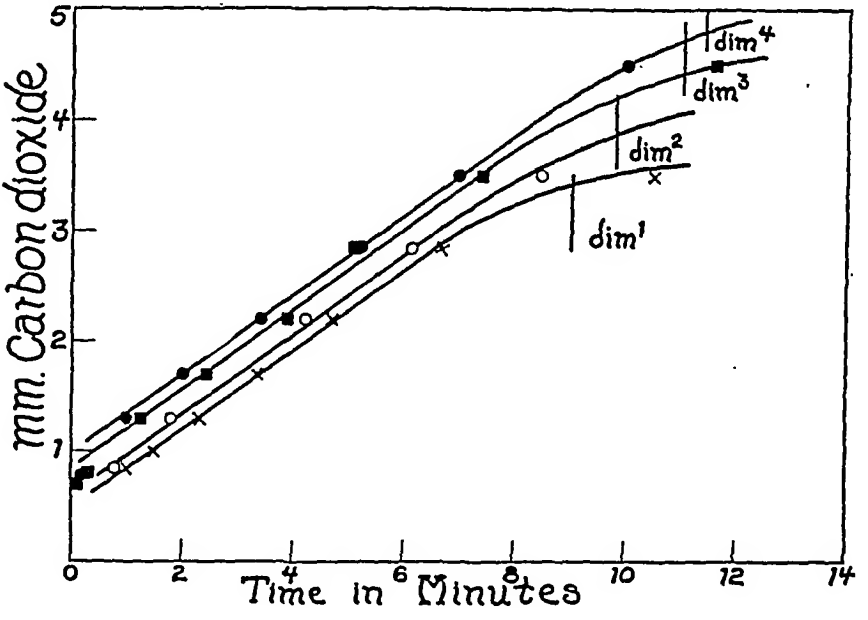


FIG. 2. Carbon dioxide production in a suspension of luminous bacteria. (Four determinations.)

to near the point of dimming, as one should expect if luminous bacteria consume oxygen independently of the concentration down to a low value of oxygen pressure. Before the point of dimming a slowing of the rate of carbon dioxide production occurs, comparable to the decrease of oxygen consumption following the lowering of oxygen to a point permitting incomplete activity of the respiratory mechanism of the cell.

In Fig. 2 a progressive increase in the time required for the dimming of a suspension of bacteria is indicated. Bacteria present in the

suspension gradually die and reduce the number of active cells. This will in turn decrease slightly the rate of oxygen consumption, allowing a longer time to be required for the reduction of the oxygen content to a point permitting dimming of the suspension because of lack of oxygen. An actual decay of the luminesence also occurs, even when the cells are adequately aerated. Hence, samples from the same suspension when repeatedly used for determinations will show a progressive

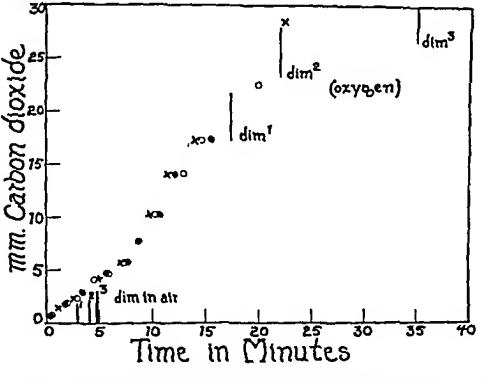


FIG. 3. Carbon dioxide production by luminous bacteria saturated with air and pure oxygen. (Three determinations.)

increase in the time for dimming. In very old suspensions a dim glow will be maintained for a remarkably long time.

Samples for carbon dioxide determinations were taken from a stock suspension that was at all times kept in equilibrium with the air and free of accumulating carbon dioxide by a stream of air continually passing through. If this stream of air is replaced by pure oxygen, it is possible to obtain a complete saturation of the suspension with pure oxygen instead of air. This was done in some cases, bringing the suspension into equilibrium with five times the amount of oxygen present in air. A five-fold increase in the time required for the dimming of the suspension would then be expected if the bacteria consume

oxygen at a constant rate independent of the pressure down to a low value of oxygen. Carbon dioxide should be produced in large quantities if all the oxygen in the suspension is consumed. This is approximately what does occur, as illustrated in Fig. 3.

Stephenson and Whetham[29b] have measured the carbon dioxide production of *Bacillus coli communis* when in equilibrium with air and oxygen and have obtained similar results.

Relative Time for the Dimming of a Suspension of Luminous Bacteria Saturated with Air and Oxygen

Determination	No. XII (air)	No. XII (saturated with oxygen)
1	3.10 minutes	17 minutes
2	4.05 "	22 "
3	5.00 "	35 "

It will be seen that in the last determination the time for dimming is *more* than five times that required for the dimming when in equilibrium with air. This is due to the reduced respiration of the bacteria when in contact with a very high concentration of oxygen, and to possible injury by high tensions of oxygen as indicated by Adams[1] and also by my own manometric experiments.

In every instance following carbon dioxide determinations in the above experiments, the suspension of luminous bacteria returned practically to its original pH on aeration.

The Effect of Oxygen Tension upon Oxygen Consumption as Determined by Manometric Methods

In the experiments of Callow[6] respiration of bacterial suspensions was successfully measured by manometric methods. In the present experiments the Thunberg-Winterstein microrespirometer was used in the form designed by Fenn,[7a] with additional modifications to permit the passage of a gas mixture into the respiratory chamber, directly above the suspension of bacteria.

By the use of a system of flow-meters, a mixture of pure nitrogen with air can be made in such a fashion that it is possible to obtain practically any desired oxygen concentration in the total mixture of gas. Pure nitrogen was obtained by passing the commercial gas over hot copper in an electric furnace. The gas was

conducted to the flow-meter through a tight system of glass and lead tubing, sealed at all joints with DeKhotinsky cement. Air was led from a second flow-meter and the gases mixed at a stop-cock connecting the two flow-meters and carried through a system of lead tubing to the water-bath containing the micro-respirometer. A complete diagram of the system is shown in Fig. 4. There was no leakage of gas or possible diffusion of oxygen into the system at any point. The only rubber connection was made very thick and extended only 2 mm. between the lead tubing and the glass tubing of the respirometer. The flow-meters were carefully calibrated for air and pure nitrogen, adjustments in pressures of the manometers giving very definite rates of flow through the capillaries of the flow-

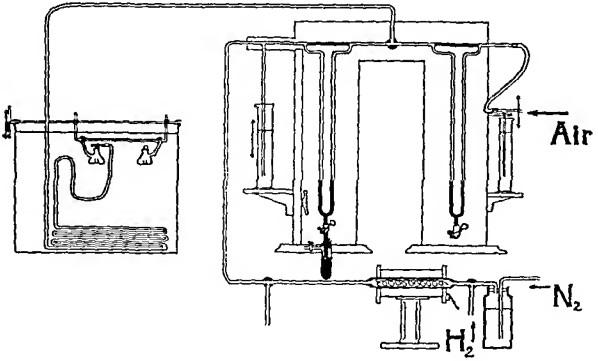


FIG. 4. Diagram of complete apparatus for determination of oxygen consumption with various gas mixtures containing partial pressures of oxygen, together with an arrangement for purification of gases.

meters. In the flow-meter conducting pure nitrogen, even the fluid of the manometer was thoroughly shaken with the pure gas before each experiment to drive off dissolved oxygen. At the microrespirometer the gas was brought into equilibrium with the bacterial suspension by a thorough shaking of the entire instrument with the mixture of gas passing through.

It was possible to make quick changes from one gas mixture to another and satisfactory determinations of oxygen consumption were easily made by this method. In each case a preliminary measurement of the rate of oxygen consumption of the particular suspension of bacteria was made in air before the suspension was subjected to a gas mixture containing only a low partial pressure of oxygen. After determinations of the rate of oxygen consumption in one or two gas

mixtures, the suspension was again brought into equilibrium with air and a check determination made to indicate if there had been a loss of respiratory activity due to decrease in the number of active cells during the course of the experiment. In each case a return to the original rate in air was made by the experimental suspension of the organisms. The suspensions were greatly diluted by phosphate buffer solution of pH 7.0, and brought into equilibrium with air by thorough shaking before a sample (1 cc.) was introduced into the respiratory chamber of the microrespirometer. The whole apparatus was rocked mechanically in the water-bath, the temperature change varying no more than 0.2 of a degree during 8 hours. The speed of rocking made no difference in the rate of movement of the indicator

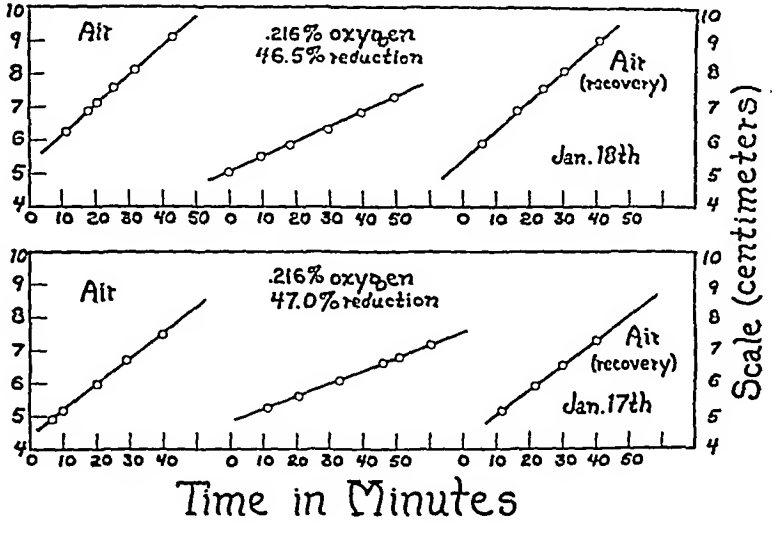


FIG. 5. Two microrespirometer determinations of oxygen consumption with equal partial pressures of oxygen.

drop of kerosene in the capillary, so long as enough movement was maintained to keep the bacterial suspension in equilibrium with the gas mixture as determinations were being made.

When gas mixtures containing the same partial pressure of oxygen were brought into equilibrium with different suspensions of bacteria, respiring at different rates in the respirometer, the percentage reduction of the respiration was nearly identical for the same gas mixtures, although the total amount of oxygen consumed by the two suspensions differed widely. This occurred in every case. An example of two determinations with different suspensions of bacteria, but identical gas mixtures is given in Fig. 5. The partial pressure of oxygen in the

gas mixtures was 1.64 mm. Hg (0.216 per cent). The reduction of respiration in the two cases is in almost exact agreement. Equally close determinations with other suspensions and other gas mixtures were made, although the agreement was less marked when only very minute traces of oxygen were present.

The value of 0.26 per cent by volume or 1.97 mm. Hg partial pressure of oxygen has been given as the point at which luminesence of luminous bacteria just begins to dim. This is in agreement with observations made during respiration in the present experiments.

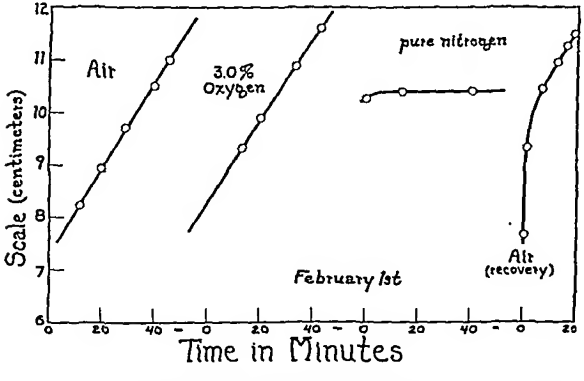


FIG. 6. Effect of pure nitrogen on oxygen consumption by luminous bacteria as measured in the microrespirometer.

When a gas mixture containing a partial pressure of oxygen equivalent to that in air (152 mm.) is brought into equilibrium with a bacterial suspension in the respirometer, there will be no increase in the rate of oxygen consumption over that at 22.80 mm. oxygen. If, however, the bacterial suspension is brought into equilibrium with pure oxygen, the rate of oxygen consumption will greatly diminish and no recovery to the original rate occurs when the suspension is again returned to air. The cells are irreparably injured by very [illegible] pressures of oxygen. This is in general agreement with the

obtained in experiments on carbon dioxide production in equilibrium with pure oxygen.

When the bacterial suspension is brought into equilibrium with pure nitrogen, the respiratory activity quickly ceases, and no recovery occurs until the suspension is again brought into contact with air, whereupon an oxygen debt is indicated as the drop in the capillary of the instrument will move rapidly across the scale, finally falling in rate of movement to that previously obtained in air.

It has been indicated previously that after luminous bacteria have been in the absence of oxygen for some time, upon being readmitted to air they will luminesce with increased brightness for a very short period. This has occurred at the moment the bacterial suspension in the respirometer has been returned to air, and an increased movement of the drop indicates a portion of the greatly increased rate of oxidation following a period in the absence of oxygen.

From the equation for calculating adequate oxygen requirement for nerve given by Gerard[8] and Fenn,[7a] Harvey, in a recent paper[11c] has given the following relation for a bacterium 1.1μ in diameter, and 2.2μ in length:

$$C_o = \frac{Ar^2}{5D}$$

for the calculation of the oxygen pressure necessary to permit oxygen supply throughout a short cylinder such as a luminous bacterium, when A is oxygen consumption of the bacteria in cubic centimeters of oxygen per gram of bacteria per minute, r is the radius of the cylinder, and D is the diffusion coefficient for oxygen for the bacteria in cubic centimeters of oxygen diffusing per square centimeter with a pressure gradient of 1 atmosphere per centimeter, the assumption being made that oxygen consumption is independent of oxygen concentration at every partial pressure of oxygen. The calculated value for C_o obtained from Harvey's (1928) previous measurements of respiration comes out 1.53×10^{-5} atmosphere* of oxygen at the surface of the bacterium to maintain adequate respiration. This theoretical value

* Dr. Harvey informs me that this value, 1.53×10^{-5} atmosphere, should not have been corrected for solubility of oxygen in sea-water as was done in his paper.

is indeed far from the actual value, for as indicated in the curve for per cent of respiration in Fig. 7, the *observed* limiting value for adequate respiration is near 0.03 atmosphere, or 22.80 mm. Hg oxygen. All pressures above this value to equilibrium with air, permit a constant and maximum rate of oxygen consumption and at every partial pressure below this value there occurs a decrease in respiratory rate. Oxygen consumption is not independent of oxygen pressure over the

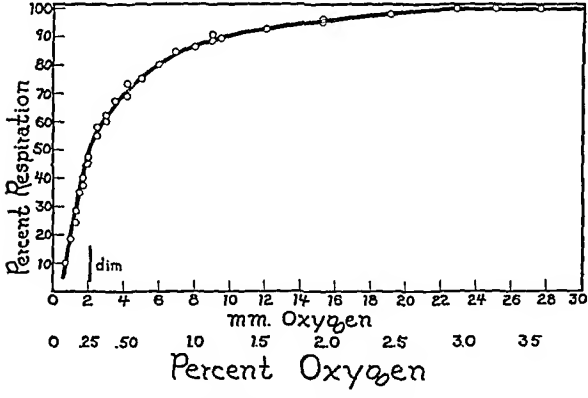


FIG. 7. The effect of oxygen tension upon oxygen consumption as measured by manometric methods.

whole range of respiratory activity, and the formula does not apply to luminous bacteria because of this assumption in the equation that oxygen consumption is always independent of oxygen pressure. The luminous bacteria are so small that the oxygen collecting at the catalytic surface of the oxidation mechanism of the cell becomes the limiting factor determining the rate of oxygen consumption rather than the oxygen diffusing into the cell.

When the partial pressure of oxygen in the gas mixtures in equilibrium with the suspension of bacteria are lowered from a value for adequate respiration, the decrease in respiratory activity does not

diminish in proportion to oxygen pressure over the whole range of oxygen concentrations. Oxygen consumption becomes about proportional to oxygen pressure only at low concentrations of oxygen following the dimming of a suspension of bacteria, when the respiration rate is reduced one-half. The curve for the reduction of respiration suggests adsorption of the gas in solution at a catalytic surface, reaching a saturation at approximately 22.80 mm. oxygen (0.03 atmosphere), at which an adequate and constant rate of respiratory activity is maintained. This is indeed similar to what has been observed in the case of inorganic catalysts, adsorbing a unimolecular layer of a gas, whose surfaces become unable to adsorb more molecules when once the surface capable of adsorption activity is covered. The per cent of maximum respiration of the bacteria will depend on the fraction of the oxidative catalyst of the cell covered by adsorbed molecules of oxygen, which in turn will depend on the rates at which oxygen is adsorbed and freed from the surfaces with the changing partial pressure of the oxygen in solution about the cells.

The curve for the reduction of respiration with decrease in oxygen pressure (Fig. 7) is exactly similar to well-known curves for the amount of gas adsorbed on catalytic surfaces which evaporate molecules with decrease in pressure.

Langmuir[18a] has discussed the relation of the extent of adsorption at various pressures of gas on a uniform surface capable of no further adsorption activity when completely covered by a unimolecular layer of adsorbed molecules, but which frees molecules of gas from the surface with decrease of pressure in agreement with an equilibrium:

$$k_1 p\,(1 - \theta) = k_2 \theta$$

when θ is the fraction of adsorbing surface covered, $(1 - \theta)$ the fraction of surface bare of adsorbed molecules, p the gas pressure, and k_1 and k_2 velocity constants characteristic of rates of condensation and evaporation from the surface respectively. If θ is considered as per cent of respiration in the curve of Fig. 7, and $(1 - \theta)$ as per cent reduction of respiration, the same relations hold, and θ will be nearly proportional to the pressure only at low pressures of oxygen, and practically independent of the pressure near saturation of the oxidative catalyst of the cell, at which point the reaction becomes one of "zero-order,"

perfectly independent of increasing gas pressures. At this high pressure of oxygen near saturation, $(1 - \theta)$ will be of small value, but will vary inversely as the pressure. The curve in Fig. 7 is a typical adsorption type, and is of the same form as curves from the data of Langmuir for adsorption of oxygen and nitrogen at mica and glass surfaces, and agrees with the form of those given by Pease[25] for adsorption of a number of gases at copper surfaces. I regard the data obtained for oxygen consumption at various pressures as supporting the view that the respiratory catalysts in the luminous bacteria are acting in a manner similar to those used in inorganic oxidations.

SUMMARY

1. The respiration of luminous bacteria has been studied by colorimetric and manometric methods.

2. *Limulus* oxyhaemocyanin has been used as a colorimetric indicator of oxygen consumption and indicator dyes were used for colorimetric determination of carbon dioxide production.

3. The Thunberg-Winterstein microrespirometer has been used for the measurement of the rate of oxygen consumption by luminous bacteria at different partial pressures of oxygen.

4. The effect of oxygen concentration upon oxygen consumption has been followed from equilibrium with air to low pressures of oxygen.

5. Luminous bacteria consume oxygen and produce carbon dioxide independent of oxygen pressures from equilibrium with air (152 mm.) to approximately 22.80 mm. oxygen or 0.03 atmosphere.

6. Dimming of a suspension of luminous bacteria occurs when oxygen tension is lowered to approximately 2 mm. Hg (0.0026 atmosphere) and when the rate of respiration becomes diminished one-half.

7. Pure nitrogen stops respiratory activity and pure oxygen irreversibly inhibits oxygen consumption.

8. The curve for rate of oxygen consumption with oxygen concentration is similar to curves for adsorption of gasses at catalytic surfaces, and agrees with the Langmuir equation for the expression of the amount of gas adsorbed in unimolecular layer at catalytic surfaces with gas pressure.

9. A constant and maximum rate of oxygen consumption occurs in

small cells when oxygen concentration becomes sufficient to entirely saturate the surface of the oxidative catalyst of the cell.

I wish to express my great debt to Prof. E. N. Harvey who first suggested this problem, for his advice, criticism, and encouragement, and also to Dr. R. N. Pease, of the Department of Chemistry, Princeton University, for some information regarding adsorption phenomena.

LITERATURE

1. Adams, A., *Biochem. Jour.*, 1912, **6**, 297.
2. Amberson, W. R., *Biol. Bull.*, 1928, **55**, 79.
3. Amberson, W. R., Mayerson, H. S., and Scott, W. J., *J. Gen. Physiol.*, 1924, **7**, 171–176.
4. Barratt, J. C. Wakelin-, *Zeit. allg. Physiol.*, 1905, **5**, 66.
5. Beijerinck, W. M., *Neerland. Arch. Sci. Exactes et Nat.*, Haarlem, 1889, **23**, 416–427.
6. Callow, A. B., *Biochem. Jour.*, 1924, **18**, 507.
7a. Fenn, W. O., *J. Gen. Physiol.*, 1926–27, **10**, 767.
b. ———, *Amer. J. Physiol.*, 1927, **80**, 327.
c. ———, *Amer. J. Physiol.*, 1928, **84**, 110.
8. Gerard, R. W., *Amer. J. Physiol.*, 1927, **82**, 381.
9. Haas, A. R., *Science*, n. s., 1916, **44**, 105.
10. Hall, F. G., *Amer. J. Physiol.*, 1929, **88**, 212.
11a. Harvey, E. N., *J. Gen. Physiol.*, 1918, **1**, 133.
b. ———, *J. Gen. Physiol.*, 1925, **8**, 98–108.
c. ———, *J. Gen. Physiol.*, 1928, **11**, 469–475.
d. ———, Nature of Animal Light. Philadelphia, 1920.
e. ———, and T. F. Morrison, *J. Gen. Physiol.*, 1923, **6**, 13–19.
12. Henderson and Cohn, *Proc. Nat. Acad. Sci.*, 1916, **2**, 618.
13. Henze, M., *Biochem. Zeit.*, 1910, **26**, 255–278.
14. Hill, S. E., *Biol. Bull.*, 1928, **55**, 3, 143.
15. Hinschelwood, C. N., Kinetics of Chemical Change in Gaseous Systems. Oxford, 1926.
16. Krogh, A., *J. Physiol.*, 1919, **52**, 391.
17. Kolthoff and Furman, Indicators. New York, 1926.
18a. Langmuir, Irving, *J. Am. Chem. Soc.*, 1916, **38**, 2221.
b. ———, *J. Am. Chem. Soc.*, 1918, **40**, 1361.
19. Lund, E. J., *Amer. J. Physiol.*, 1918, **45**, 351–364.
20. Meyer, A., Die Zelle der Bakterien. Jena, 1912.
21. Migula, W., System der Bakterien. Jena, 1900.
22. Nomura, S., *Sc. Rep. Tohoku Univ.* (4), 1926, **2**, 133.
23. Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, **1**, 167.

24. Pantin and Hogben, *J. Mar. Biol. Assn. United Kingdom*, 1925, 23, 4, 970.
25. Pease, R. N., *J. Am. Chem. Soc.*, 1923, 45, 2296.
26a. Pütter, A., *Arch. ges. Physiol.*, 1917, 168, 491–532.
b. ———, *Arch. ges. Physiol.*, 1924, 204, 94–126.
27. Redfield, Coolidge, and Hurd, *J. Biol. Chem.*, 1926, 69, 2, 475.
28. Saunders, J. T., *Proc. Cambridge Phil. Soc. Biol. Section*, 1923, 1, 43–48.
29a. Stephenson and Whetham, *Proc. Roy. Soc.*, 1923, 95, 200.
b. ———and———, *Biochem. Jour.*, 1924, 18, 498.
30. Tashiro, S., *14th Yr. Bk. Carn. Inst., Wash.*, 1915–16.
31. Thunberg, T., *Skan. Arch. Physiol.*, 1905, 17, 74–85.
32. Taylor, H. S., Treatise on Physical Chem. New York, 1925.
33. Wachsendorff, T., *Zeit. allg. Physiol.*, 1911, 13, 105–10.
34a. Warburg, O., *Zeit. f. Physiol. Chem.*, 1908, 57, 1–16.
b. ———, *Zeit. f. Physiol. Chem.*, 1914, 92, 231.
c. ———, *Arch. ges. Physiol.*, 1914, 158, 189–208.
35. Winterstein, H., *Arch. Fisiol. Firenze*, 1909, 7, 33–40.

NOTE ON THE NATURE OF THE CURRENT OF INJURY IN TISSUES

By W. J. V. OSTERHOUT and E. S. HARRIS

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, May 10, 1929)

Previous workers have found the current of injury uniformly negative but experiments on *Nitella*[1] indicate that it can be made either positive or negative according to the method of treatment. It seemed desirable to inquire whether this divergence could be explained, in part at least, by the fact that we employed single cells while previous workers have investigated tissues.

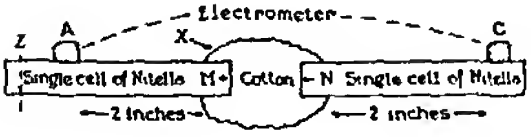


FIG. 1.

FIG. 1. Diagram to show the arrangement of an experiment. Two cells from different plants are placed in contact with a piece of absorbent cotton, *X*, and we then lead off from *A* and *C*. The letters *M* and *N* designate the ends of the cells. One cell is cut at *Z*.

By way of introduction let us consider an experiment[2] arranged as in Fig. 1 which shows two cells of *Nitella* taken from two separate plants and placed in contact with a piece of absorbent cotton. If we place 0.001 M KCl at *A*, and *C*, the cotton being wet with the same solution, we find on cutting the left-hand cell at *Z* that the potential difference of *A* (recorded with reference to *C*) becomes much more negative (Fig. 2), then quickly becomes more positive, after which it gradually approaches zero. This behavior is like that of single cells as described in former papers (in which case the experiment was arranged as shown in Fig. 3).

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

[2] The material and technique were as described in previous papers unless otherwise stated. *Cf.* (1) and *J. Gen. Physiol.*, 1927–28, **11**, 391. The experiments were carried out at room temperature averaging about 22° or 23°C.

There is no essential difference between these two cases, because, although the circuit in Fig. 3 passes through A and C and that in Fig. 1 includes[3] A, M, N, and C, the electromotive forces at N and C usually cancel out since they are opposite and almost equal, and only A and M are altered and they may be regarded as corresponding to A and C in Fig. 1. . . . . .

The situation is different when the experiment is arranged as in Fig. 4. Here we have two cells in their natural union, which consists of a cell wall (W), as shown in Fig. 5; this is only a few microns in

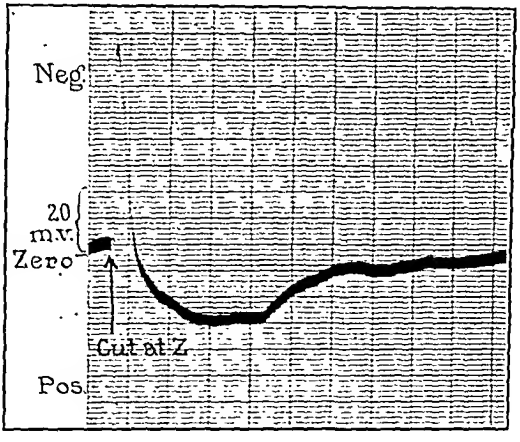


FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at A, C, and X. When the left cell is cut at Z the curve (which records the state of A with reference to C) shows that A becomes more negative, then more positive, and that the electromotive force then approaches zero. The vertical lines represent 5-second intervals. Selected as typical from 10 experiments.

thickness and in this case is imbibed with tap water. If the left-hand cell is injured so that sap[4] comes out at M, diffuses through the cell

[3] M and N represent the protoplasmic layers at the ends of the cells.

[4] The sap is equivalent in these experiments to 0.05 M KCl. *Cf.* (1). A good method of observing the coming out of sap at M is to arrange an experiment as in Fig. 4 with an additional contact at a spot, B, a little to the right of N. We put 0.001 M KCl at A and B and sap or artificial sap at C. On cutting at Z the A to C curve becomes negative and then positive after which it slowly rises to zero as sap comes in contact with N but the B to C curve does not change unless sap diffuses along to B.

wall,[5] and comes in contact with N it is clear that there is a greater difference between N and C (which is in contact with a cell wall imbibed with 0.001 M KCl). When all the E.M.F. has disappeared from the protoplasm of the cell at the left[6] (as the result of cutting at Z) the positive current will tend to flow from C (in contact with 0.001 M KCl) through the electrometer and the cell at the left (which now acts merely as a conductor) to N which is in contact with sap or a dilute sap (which acts like a solution of KCl more concentrated than 0.001 M). This

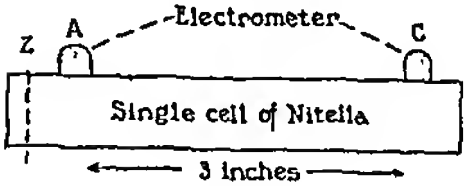


FIG. 3.

FIG. 3. Diagram to show the arrangement of an experiment. We lead off from A and C. The cell is cut at Z.

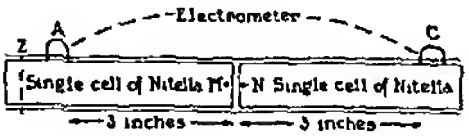


FIG. 4.

FIG. 4. Diagram to show the arrangement of an experiment. Two cells are employed, their natural union being left intact. We lead off from A and C and cut at Z. The letters M and N designate the ends of the cells

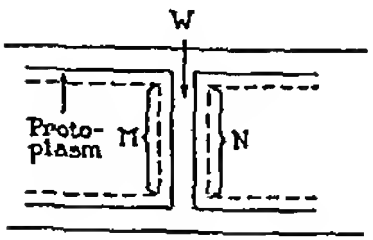


FIG. 5.

FIG. 5. Enlarged view of the point of union of the two cells shown in Fig. 4. The letter W designates the cell wall: inside this is a delicate layer of protoplasm surrounding the sap of the large central vacuole. The letters M and N designate the ends of the cells (as in Fig. 4).

gives a negative current of injury which is wholly due to the uninjured cell and which may last a long time (*i.e.*, until the cell at the right begins to lose its E.M.F. as the result of injury). Possibly this is the sort of negative current of injury observed in some cases by workers who employ groups of small cells. It is quite different from the negative current of injury in a single cell of *Nitella* which usually

[5] The cell wall is very permeable.

[6] There is some P.D. due to the cell wall which would in the present case tend to make A appear somewhat more negative than it actually is. *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, 12, 761.

lasts only a few seconds (*cf.* Fig. 2) after which the cell dies and the protoplasm[6] soon loses its E.M.F.

We assume that if sap did not come out at M[7] we should get a curve like that in Fig. 2, *i.e.*, the changes in the circuit would be confined to A and M: but if sap exudes at M and comes in contact with N the curve will tend to reach a fixed negative value, the time

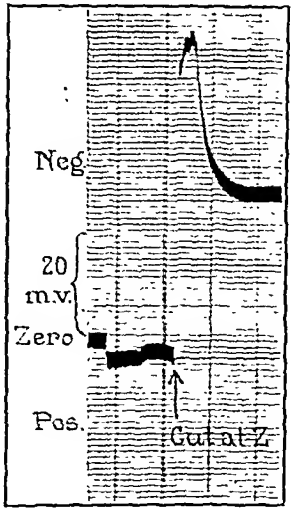


FIG. 6.

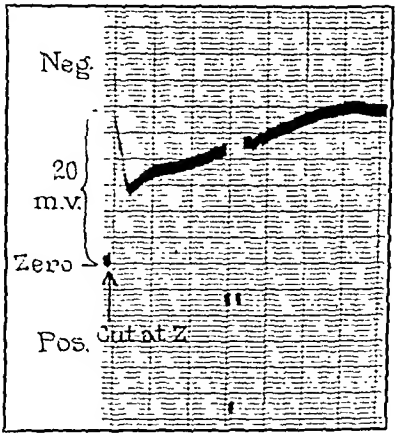


FIG. 7.

FIG. 6. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.001 M KCl at A and C. When the left cell is cut at Z the curve (which records the state of A with reference to C) shows that A becomes more negative after which the E.M.F. falls to a fixed value which is regarded as due to the coming out of sap at M (Fig. 5) which affects N, making it more negative (*cf.* Fig. 8*a*). The vertical lines represent 5-second intervals. Selected as typical from 40 experiments.

FIG. 7. Like Fig. 6 but showing a different result which is regarded as due to the slower exit of sap (*cf.* Fig. 8*b*). Selected as typical from 39 experiments. (Test for reversibility at about 16 seconds.)

depending on the speed with which the sap diffuses across the cell wall from M to N. That this time is variable is evident from Figs. 6 and 7.

[7] At the ends of the cells (*i.e.*, near M and N) there are a few very small cells exterior to the large cells forming the axis of the plant but owing to their small size it is not believed that they affect the observed P.D.

If the cell wall is imbibed with 0.001 M KCl the situation may be represented diagrammatically as in Figs. 8 *a* and *b*, where the curve[8] labelled *A* to *M* represents the P.D. of *A* with reference to *M*, and the P.D. of *N* with reference to *C* is represented by the curve labelled *N* to *C*. The observed P.D. between *A* and *C* (labelled *A* to *C*) may be regarded as the sum of these two curves and may take a variety of forms.

If before performing the experiment we apply 0.05 M KCl at the joint so that the cell wall between *M* and *N* becomes imbibed with it we may get such a curve as that shown in Fig. 9 and the situation may

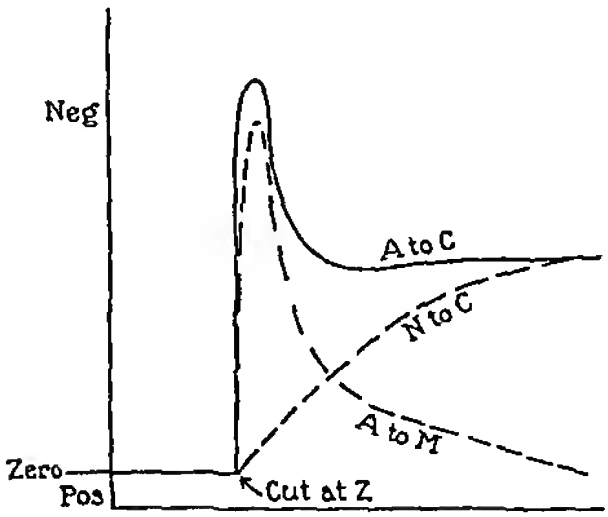


FIG. 8 *a*.

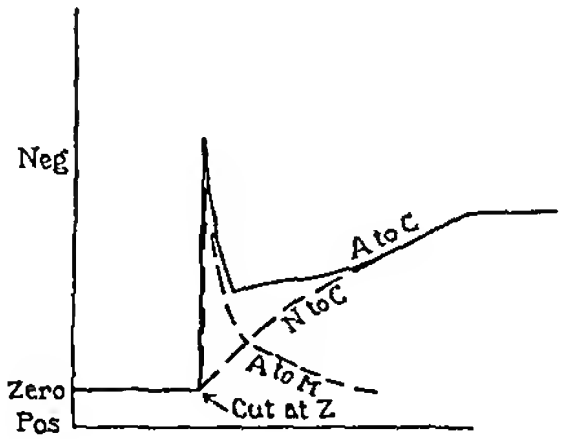


FIG. 8 *b*.

FIG. 8, *a* and *b*. Hypothetical diagram of the changes in P.D. following a cut (at *Z*, Fig. 4) when the cell wall is imbibed with 0.001 M KCl and this solution is also applied at *A* and *C*. The P.D. of *A* with reference to *M* (*cf.* Fig 4) is represented by the curve "*A* to *M*;" that of *N* with reference to *C* by the curve "*N* to C." The observed P.D. ("*A* to *C*") is the sum of these two curves. Figs. 8 *a* and 8 *b* represent two forms of such curves (*cf.* Figs 6 and 7).

be represented as in Fig. 10. The *A* to *M* curve is positive at the start and the *N* to *C* curve is negative· here too it is found that the curve may take various forms, one of which is indicated in the diagram.

In order to see how far this applies when a larger group of cells is

[8] The curves *A* to *M* in Figs. 8*a* and 8*b* are taken from actual curves obtained in cutting single cells (arranged as in Fig 3) with 0.001 M KCl at *A* and sap or 0.05 M or 0.1 M KCl at *C*. (*Cf.* Osterhout, W. J V, and Harris, E. S., *J. Gen Physiol.*, 1928-29, 12, 355.) In some cases such curves after passing through a negative phase become positive before reaching final equilibrium at zero.

involved experiments were made in the manner shown in Fig. 11. Bundles of plants were employed, the ends of the bundle being allowed

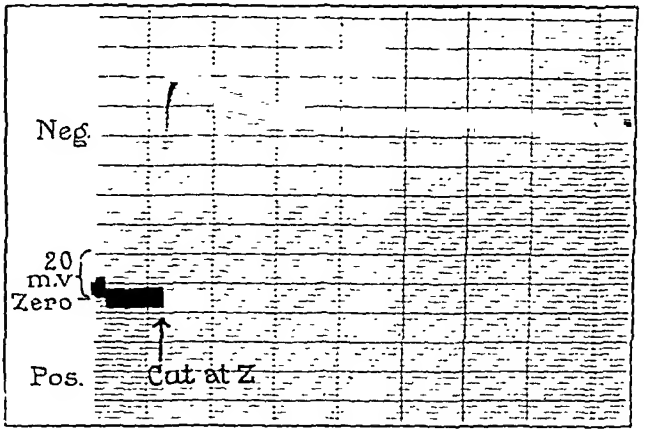


FIG. 9. As in Fig. 6 but the cell wall which separates the cells is imbibed with 0.05 M KCl. Selected as typical from 15 experiments.

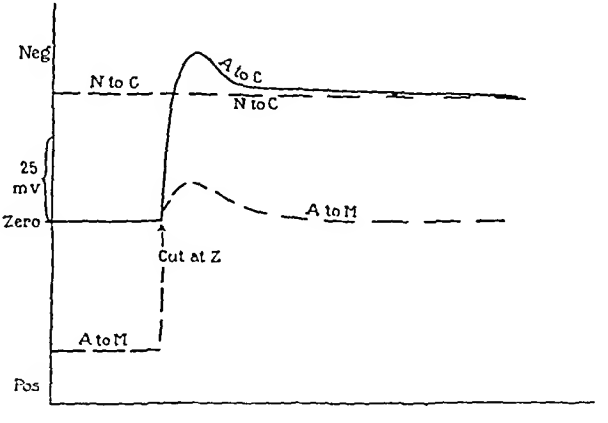


FIG. 10. Hypothetical diagram of the changes in P.D. following a cut (at *Z*, Fig. 4) when the wall separating the two cells is imbibed with 0.05 M KCl and 0.001 M KCl is applied at *A* and *C*. The P.D. of *A* with reference to *M* (*cf.* Fig. 4) is represented by the curve "*A* to *M*," that of *N* with reference to *C* by the curve "*N* to *C*." The observed P.D. "*A* to *C*" is the sum of these two curves. (*Cf.* Fig. 9.)

to dip into two dishes, *A* and *C*, in which were calomel electrodes (the cut was made at *Z*). With *A* and *C* filled with 0.001 M KCl we obtain

curves similar to that in Fig. 12 and the negative current of injury persists for many minutes.

It would seem that if our conception of the process is correct it should enable us to predict what will happen with other concentrations, for example, using the arrangement shown in Fig. 1 with 0.1 M KCl at *A* and *C* and with the cotton at *N* soaked with 0.1 M KCl, we should expect a curve like that obtained with a single cell,[9] since

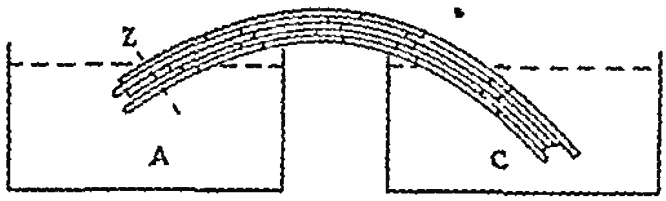


FIG. 11. Diagram to show the arrangement of an experiment in which a bundle of plants was used, the ends of the bundle dipping into the vessels *A* and *C*. The bundle was cut at *Z*.

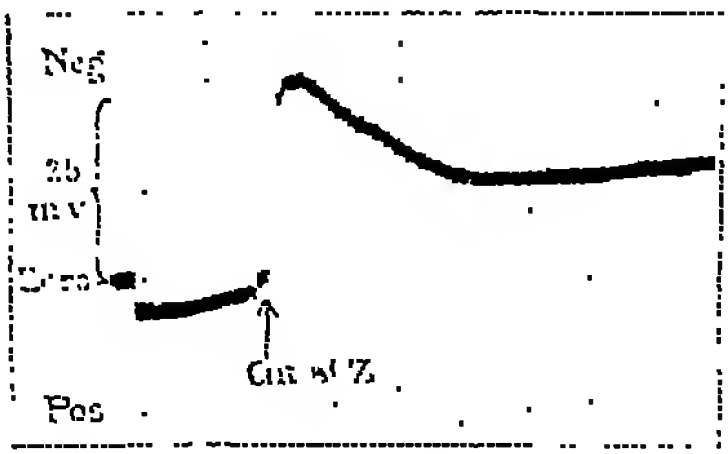


FIG. 12. Photographic record of P D., the experiment being arranged as in Fig. 11 with *A* and *C* filled with 0.001 M KCl. The vertical lines represent 5-second intervals. Selected as typical from 5 experiments.

the coming out of sap would not affect the result and at the end we should be leading off at two points, *N* and *C*, both in contact with 0.1 M KCl. This is the case as is shown by Fig. 13.

Using the arrangement shown in Fig. 4 we should expect a similar curve at the start but later on, after the E.M.F. of the cell at the left

[9] Even with single cells there is a good deal of variation in the time required for the curve to rise to zero.

has disappeared (as the result of cutting at Z) and sap comes out of M into the cell wall (which is imbibed with tap water) and reaches N the positive current will tend to flow through the electrometer from N (now in contact with sap or dilute sap) to C (in contact with 0.1 M KCl) and as the curve records the P.D. of N with respect to C

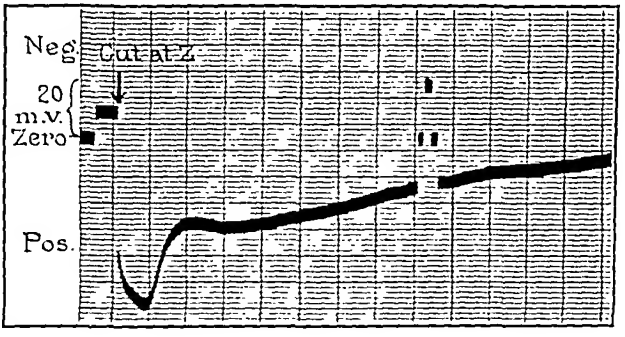


FIG. 13. As in Fig. 2 but with 0.1 M KCl at A and C. Selected as typical from 10 experiments. Test for reversibility at about 45 seconds.

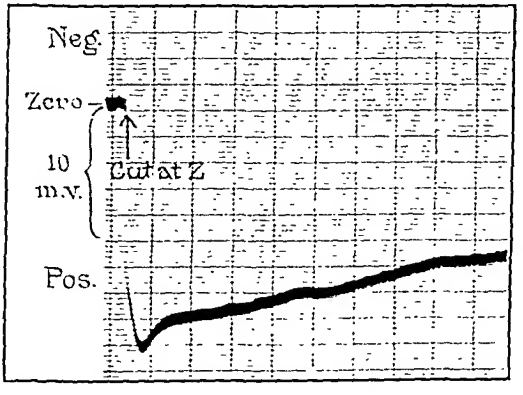


FIG. 14. As in Fig. 6 but with 0.1 M KCl at A and C and with the wall between M and N imbibed with tap water. Selected as typical from 45 experiments.

we shall expect the curve to remain positive. The more slowly the sap comes out of M the more slowly the curve will rise toward zero (this varies greatly) but even when the end wall becomes completely imbibed with sap we shall expect the curve to remain somewhat positive since sap in these experiments is equivalent to 0.05 M KCl.

That these expectations are realized is evident from Fig. 14. In this case the presence of a second cell tends to prolong the positive phase of the current of injury. The result is much the same when we employ the arrangement shown in Fig. 11.

It is evident that whenever the fluid bathing the exterior of the cell is less "negativating" than the fluid contained in the cell (as is the case in *Nitella* when 0.001 M KCl is applied to the exterior) and we lead off from the injured cell to intact cells the action of the latter will tend to prolong in a marked degree the negative phase of the current of injury.

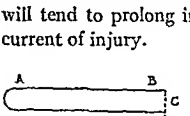


FIG. 15. Diagram of a muscle cut at one end: leading off from *A* to *C* gives more P.D. than leading off from *B* to *C*.

FIG. 15.

When we employ chloroform the injury spreads much more slowly along the cell and in many cases no spread can be detected for some minutes. In consequence the current of injury in a single cell is more lasting. This more nearly resembles the effect of cutting on muscle and nerve.[10] That there is a slow spread in muscle of the injury due to cutting is indicated by the fact that if we lead off from *A* to *C* (*Fig. 15*) we obtain a greater P.D. than in leading off from *B* to *C* but as time goes on this difference becomes less.

We may sum up by saying that whenever we can lead off from two places on the same cell (*Nitella*) or two places on a bundle of elongated cells (muscle and nerve) we may ascertain the current of injury due to the death process in the protoplasm.[11] But when we are not able

[10] The fibers of muscle and nerve differ from *Nitella* in that injury due to cutting does not spread rapidly and hence the current of injury lasts much longer. In *Nitella* there are two protoplasmic surfaces to consider. The experiments of one of us indicate the possibility that this may be true of muscle and nerve.

[11] This has been described for *Nitella* in previous papers. *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen Physiol.*, 1927–28, **11**, 673; 1928–29, **12**, 167, 355. See also Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920; Bayliss, W., Principles of general physiology, London, 1924, 4th edition; Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 1926, 6th edition.

to do this (on account of the small size of the cells) a lasting current of injury may be partly or completely due to the escape of sap which comes in contact with cells[12] which are intact or not sufficiently injured to lose their protoplasmic E.M.F. In plants the cell wall may also play a part.

SUMMARY

Leading off from two places on the same cell (of *Nitella*) with 0.001 M KCl we observe that a cut produces only a temporary negative current of injury.

If we lead off with 0.001 M KCl from any cell to a neighboring cell we find that when sap comes out from the cut cell and reaches the neighboring intact cell a lasting negative "current of injury" is produced. This depends on the fact that the intact cell is in contact with sap at one point and with 0.001 M KCl at the other (this applies also to tissues composed of small cells).

If we employ 0.1 M KCl in place of 0.001 M the current of injury with a single cell is positive (and is more lasting when a neighboring cell is present).

Divergent results obtained with tissues and single cells may be due in part to these factors.

[12] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, 12, 761.

ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. I

By W. J. CROZIER and G. PINCUS*

(*From the Laboratory of General Physiology, Harvard University, Cambridge*)

(Accepted for publication, April 10, 1929)

I

The upward geotropic progression of young rats has been formulated in two ways as a function of the inclination of the surface on which creeping takes place. The conditions necessary for the securing of significant measurements have been described, and need not be repeated.[1] The two formulations obtained are, first, an approximate descriptive relation between the mean angle of upward orientation (θ) and the slope of the surface (α), such that θ is, to a degree adequate for some purposes, a rectilinear function of $log\ sin\ \alpha$; and second, in a manner consistent with the assumption that the limiting condition for steady creeping is given by sensible equality of stretching tensions on the legs of the two sides of the body during progression, $cos\ \theta$ decreases rectilinearly as $sin\ \alpha$ increases. When genetically comparable individuals are employed, the constants in these equations are repeatedly recoverable from litters of the same inbred strain. Different inbred strains have been available, moreover, for which these equations are again applicable, but with different numerical values of the respective constants.

Geotropic animals in which the mechanics of the support of the weight of the body on an inclined plane is certainly different from that in young rodents show that the angle of oriented progression (θ) is again a function of α, but that the sort of relationship obtained changes according to the structural conditions, so that θ may be directly proportional to $sin\ \alpha$, or again to α, rather than to $log\ sin\ \alpha$, or even that $\Delta\ log\ \theta\ /\ \Delta\ \alpha = const.$ (*cf.* Wolf, 1926–27; Crozier and Stier, 1928–29; 1929–30, *a;* Kropp and Crozier, 1928–29; Kropp, 1929; Hoagland,

*National Research Council Fellow.

[1] Crozier and Pincus, 1926; 1926–27, *a*, *b*, *c*, *d;* 1927–28; Crozier, 1928

1929; Crozier, 1928; several other instances remain to be described). Consistent with these findings regarding empirical descriptions of the geotropic orientation, each of the instances cited provides its own more rational equation in terms of the limitation of oriented creeping through the distribution of tensions in the supporting musculature.

Such findings strengthen the opinion that the equations concerned may very well have a functional significance beyond their value as means of empirical description. "The genetic utilization of such differences, supposing them to be found, can provide upon the one hand a biological test of the reality of the behavior constants, and upon the other a means for the consideration of certain concrete aspects of behavior in relation to inheritance" (Crozier and Pincus, 1927–28). This amounts to an application of the atomistic conceptions of genetics in the search for a biological proof that differences recognized mathematically in the behavior of two otherwise closely comparable organisms may have a "real," that is structural or molecular, basis.

We are under very real obligation to Professor W. E. Castle for his kindness in supplying us with animals necessary for the experiments, and for his interest in the progress of the tests.

The observational basis for the theoretical superstructure employed in the present discussion has been developed in a series of papers by a number of workers in this Laboratory (*cf.* CITATIONS). Important features of our conclusions from certain of these data have been adversely criticised by Hunter (1927), Piéron (1928, *a;* 1928, *b*), and Hovey (1928)—the latter working under the direction of A. R. Moore. We are quite sure that these criticisms originate from misconceptions which are in large part not unfairly characterized as inexcusable; some of these we have indicated in previous papers (Crozier, 1928; Crozier and Pincus, 1928–29; Crozier and Navez, 1929). Both Hunter (1927) and Piéron (1928, *b*) make considerable parade of "statistical" treatment of their data drawn from experiments supposedly comparable with ours, but these writers, and Hovey (1928), carefully avoid reference to the fact that in every instance the measurements we have secured have been obtained in such a manner as to provide an "internal check" automatically emerging from the changes in the variability of the recorded magnitudes of the orientations (θ) as a function of the inclination of the creeping surface. This kind of control of the significance of the data comes about in a way which cannot possibly be influenced by the experimenter during the tests, and failure to appreciate its meaning can arise only from causes for which the present writers decline to accept responsibility.

Under conditions such as we have maintained, the specific mean magnitudes of θ, in homozygous races of rats, have been found to be quantitatively recoverable in repeated series of tests over long periods. Under conditions which many earlier trials had taught us to be unsuitable for any reliable tests, wide fluctuations in upward orientation are easily observed. The influence of light, for example, unquestionably present (together with other sources of trouble) in Hunter's (1927) experiments—and said by Hovey (1928) to be without significance!—is easily shown by such tests as the following (*cf.* also Keeler, 1927–28, with mice). On a creeping plane tilted at $\alpha = 20°$, certain individuals of our race *K* gave under weak red light mean $\theta = 44° \pm 1.9$, as compared with $44.5° \pm 3°$; $43.7° \pm 2°$; $46.1° \pm 1.6$, obtained in longer series of tests at intervals during a period of two years (the first two series are cited in Crozier and Pincus, 1926–27*a*; 1928–29; the third series is based on only twenty-two observations). As is usual at such low inclinations, creeping is slow (Pincus, 1926–27) and there is much "random movement." It is necessary to wait for the intermittent periods of active progression, in which alone is orientation evident. But when a white light of moderate intensity (approx. 1 f.c. illumination) is turned on, above and somewhat in front of the surface, creeping is for some time much faster and the random movements less in evidence. At first, θ may be almost 90°. In successive runs it slowly decreases. At the end of half an hour or more, exposure to the light being continuous, θ then becomes as low as 45°. In duplicate experiments, the mean θ obtained from such tests may be as high as 80° at $\alpha = 20°$. If the source of light be somewhat to one side of the animal, it swerves in the opposite direction, and θ may be as low as 0°; again, with exposures continuing for some time, θ slowly increases. If under dim red light a young rat be proceeding in a well oriented, straight path and a white light of even low intensity be then turned on above it and slightly to the rear, θ is sharply increased (after a measurable latent period); if the white light be then excluded, θ is seen to decrease if a brief pause or hesitation permits reorientation; in the absence of adequate lateral swerving there is no particular reason (*cf.* Crozier and Pincus, 1926–27, *a*) why the animal should change its path, since on the theory we have presented orientation in the area delimited by $+\theta$ and $-\theta$ is not geotropically constrained; this very obvious condition of the experiments seems to have been deliberately violated in Hovey's (1928) experiments, and here unquestionably accounts in part for the prevalence of high values of θ at each magnitude of α, since the rats were in this case started creeping vertically on the plane and not headed to one side. These are elementary phenomena of phototropic orientation in a situation involving competition between geotropic and phototropic vectors (Crozier and Pincus, 1926–27, *b*, *c*; Wolf and Crozier, 1927–28), coupled with the occurrence of photic adaptation. If tests are made in a room with one or more windows admitting daylight, almost any complexity of behavior may be witnessed on the inclined surface. In this connection we would emphasize the fact that the upward orientation on an inclined surface cannot possibly be regarded as due to mechanical inability of the young rat to move downward. At inclinations not too great, it is easily possible to force the animal to creep downward by

illumination, and in fact downward creeping sometimes occurs spontaneously (*cf.* Crozier and Pincus, 1928–29). We therefore reject as equally inapplicable the suggestion by Wickham (1928, p. 163) that young rodents creep on inclined surfaces because they are "afraid of falling," and that advanced by Hovey (1928), following a similar thought expressed by Hunter (1927), that only in upward progression can the animals obtain a secure footing.

The contention that the oriented upward movement of young rats upon an inclined surface is not a tropistic response because it is affected by "learning" (Hovey, 1928) is of course irrelevant even if it were true. In the paper cited no proof of learning is indeed given, because during a prolonged series of observations upon single individuals the conditions of progression change as a result of the rather rapid development of the musculature of the appendages at this period of growth (in well-nourished litters). It would be necessary to have data upon the progression of "untrained" individuals of the same effective age; it is not sufficient to compare mean angles of orientation at the beginning and at the termination of a long series of trials. Comparisons of this sort which we have made with rats and with guinea pigs show under suitable conditions no effect due to "learning." Moreover, in such experiments (Hovey, 1928) there enters the possibility of errors of a sort very clearly evidenced in experiments with ants. During an investigation of the geotropic orientation of ants of various species it was found by Dr. T. C. Barnes, and by Mr. B. F. Skinner, that when an individual of *Aphaenogaster fulva* is kept isolated from the nest and is tested at intervals during several days, the angle (θ) of oriented progression at a given slope of surface shows definite increase. But this rise in θ is also exhibited by an individual kept in isolation for a similar period without trials upon the sloping surface.

The possibility of "facilitation" effects ("learning") was very early explored in these experiments.[1] No trace of it could be found, under the conditions of our tests. But again there are misleading occurrences which require that the observer exercise reasonable intelligence. With 8 individuals, for example, tests were made at 7 values of α to determine if a second series of trials, several hours subsequent to a first series, would yield a different mean θ. It was found, using 20 observations with each individual at each slope in each series, that θ was in these 56 tests on the average 1°.32 *less* in the second series than in the first. The difference $(\theta_1 - \theta_2)$ was therefore contrary in sign to what would be expected for the effect of a valid influence of "learning." It happened that these experiments were so planned that values of θ_1 were obtained in the morning, of θ_2 in the late afternoon. While the absolute differences never exceeded $(\theta_1 - \theta_2) = -5°$ in any one case, and in the mean gave $-1°.32$, the difference is entirely consistent with the known lessened activity of the young rodent in the afternoon and evening, as contrasted with morning hours, even when external conditions have been kept constant; this has been established by Szymanski (1920, 1922), and others, and for young individuals with greater technical refinement by Dr. E. Wolf and by Dr. T. J. B. Stier in the course of investigations now in progress, in which the effects of feeding have been controlled. So far as it goes, the result of these tests merely con-

firms the indications from the application of other criteria, namely that the technic of the present experiments has been relatively free from gross sources of inadequacy. It should be added that when an additional load is carried, θ at given (low) α is increased, but that tests made immediately after the removal of the load (*cf.* last section of the present paper) show θ-values uninfluenced in any way by the preceding experience.

The considerations adduced by Piéron (1928, *a; b*) are of a somewhat different order. Arguing, reasonably enough, that if the limitation of upward orientation in such a gasteropod as *Agriolimax* (Wolf, 1926–27) is controlled by the adjustment of gravitationally induced tensions in the musculature of the body as we have considered in the case of rodents, then the amount of upward orientation should be less if the animals are put under water. However, as experiments made by one of us (W. J. C.) some years ago had shown, the slug *Limax*, as used by Piéron, is not exactly suitable for such tests. When placed on a glass surface and then lowered under water the direction of subsequent turning is largely influenced by the mode of lowering of the plate into the water, and by the relation of the aquarium to the positions of windows (although the cepahlic tentacles are not fully everted). What actually happens in such trials we shall discuss in detail elsewhere. When observational errors of these kinds are excluded, and allowance is made for the fact that the foot does not adhere to glass under water, and that the slugs do not creep, there is also in Piéron's account an apparent error of curious sort regarding the measurement of the angles of orientation. For the present, we would therefore cite the facts revealed in a long series of experiments in Cuba by one of us and Dr. A. E. Navez, employing *Onchidium* and various land snails of which the foot adheres well under water, and which creep vigorously; it is found: (1) that the "angle effect" in orientation is perfectly real, in air *or* under water; (2) that by the attachment of a cork float to the shell a form such as *Ligius* can be forced to orient *downward* under water, rather than upward as in the absence of the float; (3) that the reaction-time to geotropic excitation, obtained by a method which avoids handling, is a function of α, and of the mass of loads added to the shell (in air or in water). These findings are in perfect accord with the proprioceptive origin of geotropic orientation, and flatly contradict Piéron's contention. We are therefore quite justified in dismissing Piéron's conclusions as having no weight in this matter, since his alleged phenomena are in fact superficial accidents having no bearing upon the point at issue.

II

It should be obvious, of course, that persistent self-consistency in the performance of animals within a well-inbred line must be due to likeness of "inheritance." The two profitable questions which arise have to do with (1) the genetic behavior of recognizable differences when unlike lines are crossed, and (2) the possibility of utilizing the resulting genetic criteria for testing the functional interpretation of the

original differences. This amounts to a mode of definition of an hereditary effect, or of a gene, namely in terms of the connection between its organic expression and the values of an effective and experimentally controllable variable. There is implied in the acceptance of this procedure the view that the customary description of genic differences is imperfect. It has come to be rather generally recognized that a major problem in the theory of genetics is that of the method whereby genic effects are produced. The analytical manipulation of genic differences, however, clearly requires something more than mere description in terms of developmental rates and times of onset. It in fact necessitates the characterization of genetic contrasts in terms of magnitudes having functional dimensions. In only one group of instances have data of this sort been provided, namely in the case of eye-facet number in Drosophila; and here the analysis of the relations (to temperature, in this case), has certainly been defective.

The proposition we here advance serves to bring out one aspect of a general method of biological analysis which we believe to hold promise of wide significance. In outline, this method of defining an effect in inheritance seeks to avoid the pitfalls of the pseudo-quantitative treatment which depends upon mere differences in terms of a relationship of "*greater*" or "*less*" between two values of a certain variable; it does so by attempting to obtain the law of the relation between each expression of the variable in question and some controllable external variable. Instances will be given in which (*cf.* Crozier and Pincus, 1927–28) this kind of description can be shown not merely desirable, but *necessary*.

It is not to be taken for granted that the primary variable,—in cases such as we now chiefly discuss, the difference in geotropic behavior between two groups of young rats,— is in itself likely to be a simple thing. But we may illustrate the necessity for the introduction of an additional coördinate in such analyses by considering briefly the case previously described (Crozier and Pincus, 1927–28); here, two races of *Rattus norvegicus*, *A* and *K*, are found to differ from one another, as regards geotropic orientation, in the way demonstrated by Fig. 1. If comparisons between these two races were to be made solely at a low value of α it might be said that race *A* is more responsive, more sensitive, geotropically, since θ is there greater for *A* than for *K* (although its workable threshold α is higher); but if the comparison

were made only at high values of α the conclusion would however be exactly the reverse; and in an intermediate region the two races would appear identical. Thus it may happen, in any given case, that the organic variable may be fundamentally altered, genetically, in the sense that the *form* of its quantitative relationship to some external condition of its expression is no longer the same. But unless the law of the relationship can be adequately established over a range of values of an independent variable, this sort of contrast between two types of organisms cannot be differentiated from that in which the absolute magnitudes (of response, let us say) are altered while the form of dependence upon the experimental variable is still the same. This again may be illustrated by means of Fig. 1.

Data obtained with race *B* are chosen, from among others available, to show that the absolute amount of geotropic response may be markedly different in two kinds of individuals, (*A*, *B*), under the same conditions, although the way in which the response is affected quantitatively by increasing the magnitude of the exciting condition remains the same. Race *B* rats were from the 11th brother x sister generation of a line which before this period of closest inbreeding was loosely inbred. For the test of which the results are given in Table I, 5 individuals were used. θ, and P.E.$_\theta$, depend upon sin α in the way already described for the other races; the number of readings at one inclination varied between 51 and 62, so that in Fig. 2 the value of σ_M as a percentage of the mean has been plotted to demonstrate that in this line also the decreasing *variability* of response is sensibly a straight line function of log sin α. At $\alpha = 70°$ the results with *B* were not quite so certain as with *K* or *A*, due to a small percentage of cases in which slipping was present, and it is to be noticed that the measure of the variability of θ correspondingly shows a slight but definite rise (*cf.* also, Crozier and Oxnard, 1927–28). The weights of these rats, on the 13th–14th day after birth, when used for the experiment, were 20.5 $\pm$ 0.91 gms.; later we shall have occasion to refer to this point. The lesser relative reliability of the mean θ at $\alpha = 70°$, although in no sense serious, in this case noticeably disturbs the position of the cos θ plot (*Fig. 1*) at this value of the slope (α), since cos θ is more sensitive at the higher angles.

The two types of organic difference here diagrammed might obviously be combined in such a way as to occur simultaneously (*cf.*

data in: Crozier and Pincus, 1927–28). Another illustration of the general principle may be taken from studies on the temperature characteristics of vital processes. If the addition of a reagent induces change in the frequency of heart-beat, for example, at constant

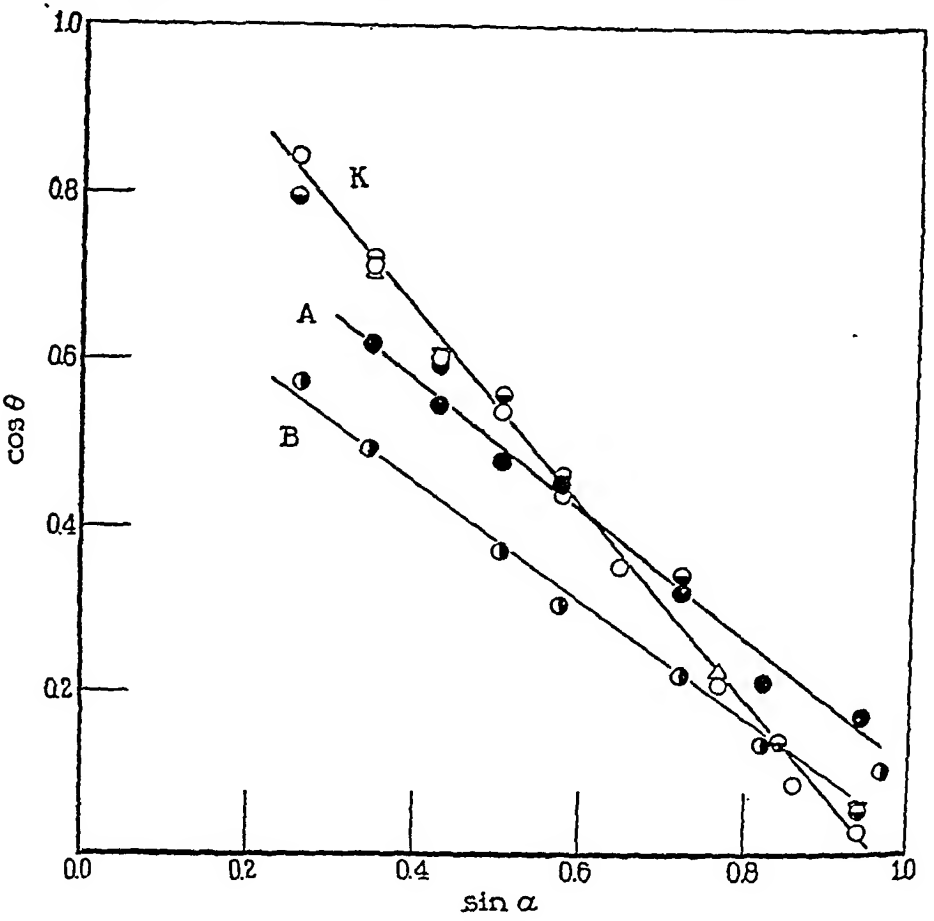


FIG. 1. Geotropic orientation of young rats of three genetically distinct races *K*, *A*, and *B*, presented in terms of the linear relationship between *cos θ* and *sin α*, where α is the slope of the surface and *θ* is the angle of oriented progression on the surface. (Four series of measurements are plotted for race *K*, to show reproducability; see text.)

temperature, we cannot tell from this alone whether the frequency has been altered without change of temperature characteristic, or that the whole basis of the relation to temperature has been changed (*cf.* Crozier 1925–26; Crozier and Stier, 1924–25, *a;* 1925–26, 1926–27;

1929–30, *b*, *etc.*); the mere statement that "the frequency has been increased" may be true in one part of the temperature range but not at all true in another, and by itself is of practically no analytical significance. But if the relation to temperature is identical in the presence of the reagent, though the absolute frequencies are no longer the same,

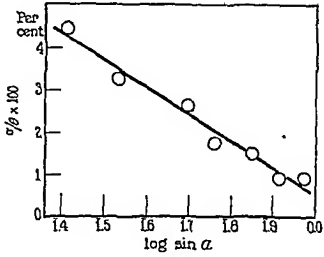


FIG. 2. Variability among measurements of θ with race *B* obeys the same rule as found with races *K* and *A*; see text.

it is clear that the mode of action of the reagent is to be described in a way which must differ materially from that required if the frequency is no longer the same kind of a function of the temperature. With regard to problems of inheritance, the point fundamentally at issue appears not unusually to be of just this kind.[2]

III

We may first consider the results of genetic tests involving races *K* and *A* in terms of the relation between $\cos \theta$ and $\sin \alpha$ (Fig. 1). On the assumption that in upward geotropic creeping the young rat orients until there is equality of stimulation by downward tensions

[2] The point we desire to make may not be completely new, but certainly it has not been expressed clearly hitherto, nor used. Thus, in discussing cases in which a particular genetic type appears different from another one only in a special environment, the best usual opinion has been (Morgan, Sturtevant, Muller, and Bridges, 1915; 1922, pp. 38 *et seq.*) that in most cases of genetic difference "the environment, being common to the two, may therefore in such cases be ignored, or rather taken for granted." In cases of the former type, it is recognized that "it is the different possibilities of reaction that are inherited." Our point

on the legs of the two sides of the body, in the direction parallel to the inclined surface, it appeared[1] that it should be found that

$$\Delta \cos \Theta / \Delta \sin \alpha = - const. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

If the difference between lines K and A, as related to the magnitude of the slope constant in Equation (1), has an approximately simple genetic basis we should expect: (*a*) possible indications of dominance

TABLE I

Mean angles of upward orientation (θ) obtained in several series of tests with young rats of lines K and A. With line K, series IV is based upon observations (*i* and *ii*) made two years after those in series I (for series III, and the data on line A, see: Crozier and Pincus, 1927–28). The inclinations of the creeping surface are given in the first column.

α Deg.	θ, degrees					
	Line K			Line A		Line B
	I	III	IV	I	II	
15	32.6±2.66	37.4 ±				55.05±1.65
20	44.5±1.01	43.70±2.05	(i) 45.4 ±1.30	51.88±0.23	52.81±1.40	60.52±1.34
25	52.9±0.97	53.75±2.24	(ii) 52.71±1.17	56.97±0.21	58.27±1.40	
30	57.4±0.98	55.98±1.72		61.39±0.18	59.28±1.26	68.24±1.22
35	64.0±0.90	62.50±1.21		63.19±0.16	63.05±1.02	72.16±0.85
40	69.8±0.82					
45		69.89±1.18		71.24±0.14	71.44±1.05	77.26±0.78
50	77.9±0.81		(i) 75.11±1.38			
55		81.80±0.81		77.69±0.16	78.52±1.02	82.05±0.51
60	84.7±0.46					
70	88.3±0.31	86.75±0.43	(ii) 86.6 ±0.51	80.17±0.10	79.54±0.73	83.98±0.53

in F_1, but, more significantly, (*b*) evidence of segregation in back crosses between F_1 and P_K and P_A. In comparing Equation (1) with the subsequent treatment, it is necessary to remark that the deviations of the data from its curve are statistically real and significant,

either instance the "possibilities of reaction" require quantitative formulation, and that such formulation necessitates statement as a function of some independent variable. Growth-curves, survivorship curves, and dubious "reaction rate" curves do not give this information. Many years ago the investigation of the temperature coefficients for frequency of heart beat in hybrids of *Fundulus* and *Menidia*, however, was undertaken by Loeb and Ewald (1911). We expect to have more to say about such cases in the near future.

though small, even for races K and A; hence we cannot pass directly from equation (1) to the differential $d\theta/d \log \sin \alpha$ subsequently used.

Line K represents about 60 generations of brother by sister matings and is identical with the King line of inbred albinos. Albinos of the

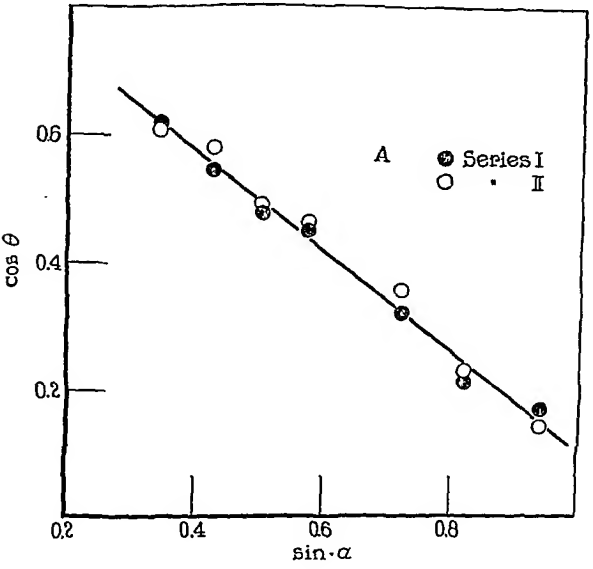


FIG. 3. Showing agreement between independent determinations of the geotropic orientation function with young rats of race A. Data in Table I (and *cf.* text).

King strain were indeed used in making the crosses for these experiments. As regards color factors, the K animals were cc RR aa hh, where c = albino, R = dark eye, a = non-agouti, and h = hooded.

The A animals were of the 10th and 12th brother x sister generation

of an inbred line of red-eyed yellow hooded animals, and as regards the color factors were CC rr aa hh. The *B* line is also red-eyed yellow hooded, and the animals tested were of the 11th brother x sister generation.

To test the reliability of the original data on the *A* line a series of experiments was undertaken with a new litter of 5 individuals (of the twelfth generation of closest inbreeding), some months subsequent to the publication of our previous paper in which the characterization of this race is given (Crozier and Pincus, 1927–28). The technique was as already described. The results are given in Table I (under *A*, Series II) and in Fig. 3. It is of interest to compare these figures with those earlier obtained. In *Series I (A)*, 12 individuals were employed, with approximately ten observations on each. In *Series II*, 5 individuals with 15 observations on each. In both cases the variability of θ declines with increase of α, and in the manner we have specified in previous papers (Crozier and Pincus, 1926–27, *a*; 1927–28). It has been pointed out (1927–28) that if two sets of tests are made, one with N individuals and *twenty* observations on each, the other with $2N$ individuals and *ten* observations on each, the measure of variability should in the former case decline twice as rapidly, as *log sin* α increases. This is in consequence of the assumption that the measure of variability $\propto 1/\sqrt{n}$, where n is the number of observations at every value of α, and also $\propto \sqrt{N}$, where N is the number of individuals which serve as separate "centers" or foci of variability of reaction. When $\text{P.E.}_\theta/\theta \times 100$ is a usable measure of the variability expressed as a function of *log sin* α, we then look for the decline of variability in our first instance to be related to that in the second by the factor $\sqrt{\frac{N}{20} \times \frac{10}{2N}}$, or in the ratio of 2:1, which was observed (Crozier and Pincus, 1927–28). This method of comparison is important, and can be generalized—roughly, it is true, but with significant results.[3]

[3] It might be objected (*cf.* Hunter, 1927; Hovey, 1928) that the frequency distributions of θ at each magnitude of α are in fact definitely skewed. To this there are several replies, not mutually exclusive. In the first place, unless number of individuals and number of observations are the same, within rather narrow limits, skewness can be introduced by differences of sensitivity (temporary or permanent) among the separate individuals (*cf.*, *e.g.*, the discussion in: Crozier and Stier,

Series I (*A*) was run in two stages, tests being made with individuals in two groups of six each, with a slight difference between the two groups. We expect that the slope of the line connecting $\text{P.E.}_\theta/\theta \times 100$ with *log sin* α to be *about* the same with the data for individuals 1–6 and 7–12. The results are given in Fig. 4. The slight age difference between the groups (1–6) and (7–12) may be responsible for the fact that the ratio of the slopes is not 1.0 but 0.83; in any case the agreement must be regarded as close. If we compare these data with those from *Series II* (*A*) (Fig. 4), we expect the slopes to be, for Series I (1–12) and Series II, in the ratio $\left(\sqrt{\frac{75}{100}}\right)\left(\sqrt{\frac{12}{5}}\right) = 1.34$. The ratio found is 1.46. Between Series I (1–6) and Series II we expect, on the same basis, a slope ratio of 1.29; that found is 1.28. We regard this sort of agreement as an excellent test of the constancy of the

---

1927–28, 1928–29; Crozier and Pincus, 1927–28). Secondly, if our conception of the situation be at all correct, it is illegitimate to group the observed θ's into size classes of the same magnitude; both as regards the grouping of mean θ's as a function of the slope of the surface, and as concerns the grouping of θ's in a frequency polygon at a given value of α, the size-classes must be arranged in a series which recognizes the way in which the variability of θ is empirically found to behave (*cf.* Crozier and Pincus, 1927–28). There are two ways in which this condition can be met. One may either arrange the θ's (at given α) in a logarithmic frequency distribution, and then deal with the *geometric mean* of the θ's; or the frequency distribution may be based upon the values of *cos* θ. In either case the asymmetry evident in arithmetic seriations of observed θ's when large numbers of readings with homogeneous material are available, sensibly disappears (as is indeed obvious to mere inspection). These tests we have in fact made long ago. Since with the arrays of θ's we have used it is found that the indicated statistical refinements make no detectable difference in the usable end result, they have been ignored. But we would stress (1) the point that when for example slipping of the animals occurs, such as causes trouble in experiments with young mice at high values of α, and is easily recognized and noted, the P. E. of θ increases (both absolutely and relatively; Crozier and Oxnard, 1927–28) while the mean θ does not increase as expected; hence the two modes of treatment of the mean θ's supply important mutual checks; and (2), more significant, that the "skewness" of the frequency distributions of θ is in fact precisely what we have reason to expect if the general interpretation we have employed be essentially sound.

of an inbred line of red-eyed yellow hooded animals, and as regards the color factors were CC rr aa hh. The *B* line is also red-eyed yellow hooded, and the animals tested were of the 11th brother x sister generation.

To test the reliability of the original data on the *A* line a series of experiments was undertaken with a new litter of 5 individuals (of the twelfth generation of closest inbreeding), some months subsequent to the publication of our previous paper in which the characterization of this race is given (Crozier and Pincus, 1927–28). The technique was as already described. The results are given in Table I (under *A*, Series II) and in Fig. 3. It is of interest to compare these figures with those earlier obtained. In *Series I (A)*, 12 individuals were employed, with approximately ten observations on each. In *Series II*, 5 individuals with 15 observations on each. In both cases the variability of θ declines with increase of α, and in the manner we have specified in previous papers (Crozier and Pincus, 1926–27, *a*; 1927–28). It has been pointed out (1927–28) that if two sets of tests are made, one with N individuals and *twenty* observations on each, the other with $2N$ individuals and *ten* observations on each, the measure of variability should in the former case decline twice as rapidly, as *log sin* α increases. This is in consequence of the assumption that the measure of variability $\propto 1/\sqrt{n}$, where n is the number of observations at every value of α, and also $\propto \sqrt{N}$, where N is the number of individuals which serve as separate "centers" or foci of variability of reaction. When $\text{P.E.}_\theta/\theta \times 100$ is a usable measure of the variability expressed as a function of *log sin* α, we then look for the decline of variability in our first instance to be related to that in the second by the factor $\sqrt{\frac{N}{20} \times \frac{10}{2N}}$, or in the ratio of 2:1, which was observed (Crozier and Pincus, 1927–28). This method of comparison is important, and can be generalized—roughly, it is true, but with significant results.[3]

[3] It might be objected (*cf.* Hunter, 1927; Hovey, 1928) that the frequency distributions of θ at each magnitude of α are in fact definitely skewed. To this there are several replies, not mutually exclusive. In the first place, unless number of individuals and number of observations are the same, within rather narrow limits, skewness can be introduced by differences of sensitivity (temporary or permanent) among the separate individuals (*cf.*, *e.g.*, the discussion in: Crozier and Stier,

Series I (*A*) was run in two stages, tests being made with individuals in two groups of six each, with a slight difference between the two groups. We expect that the slope of the line connecting $P.E._{\theta}/\theta \times 100$ with *log sin* α to be *about* the same with the data for individuals 1–6 and 7–12. The results are given in Fig. 4. The slight age difference between the groups (1–6) and (7–12) may be responsible for the fact that the ratio of the slopes is not 1.0 but 0.83; in any case the agreement must be regarded as close. If we compare these data with those from *Series II* (*A*) (Fig. 4), we expect the slopes to be, for Series I (1–12) and Series II, in the ratio $\left(\sqrt{\frac{75}{100}}\right)\left(\sqrt{\frac{12}{5}}\right) = 1.34$. The ratio found is 1.46. Between Series I (1–6) and Series II we expect, on the same basis, a slope ratio of 1.29; that found is 1.28. We regard this sort of agreement as an excellent test of the constancy of the

---

1927–28, 1928–29; Crozier and Pincus, 1927–28). Secondly, if our conception of the situation be at all correct, it is illegitimate to group the observed θ's into size classes of the same magnitude; both as regards the grouping of mean θ's as a function of the slope of the surface, and as concerns the grouping of θ's in a frequency polygon at a given value of α, the size-classes must be arranged in a series which recognizes the way in which the variability of θ is empirically found to behave (*cf.* Crozier and Pincus, 1927–28). There are two ways in which this condition can be met. One may either arrange the θ's (at given α) in a logarithmic frequency distribution, and then deal with the *geometric mean* of the θ's; or the frequency distribution may be based upon the values of *cos* θ. In either case the asymmetry evident in arithmetic seriations of observed θ's when large numbers of readings with homogeneous material are available, sensibly disappears (as is indeed obvious to mere inspection). These tests we have in fact made long ago. Since with the arrays of θ's we have used it is found that the indicated statistical refinements make no detectable difference in the usable end result, they have been ignored. But we would stress (1) the point that when for example slipping of the animals occurs, such as causes trouble in experiments with young mice at high values of α, and is easily recognized and noted, the P. E. of θ increases (both absolutely and relatively; Crozier and Oxnard, 1927–28) while the mean θ does not increase as expected; hence the two modes of treatment of the mean θ's supply important mutual checks; and (2), more significant, that the "skewness" of the frequency distributions of θ is in fact precisely what we have reason to expect if the general interpretation we have employed be essentially sound.

technic employed in the experiments, and of the homogeneity of the material constituting each set of animals tested.

It may be worth while to extend this mode of calculation to the comparison of different races as regards variability. If two races of young rats are intrinsically alike with respect to properties influencing the expression of θ as a function of α under the conditions of these experiments, then we expect the slopes of lines connecting P.E.$_\theta/\theta \times 100$ with *log sin* α to be in the ratio already given, $\sqrt{\frac{(N_1)\ (n_2)}{(n_1)\ (N_2)}}$, where N_1, N_2 are the numbers of individuals of races *1*, *2*, concerned in the tests, n_1, n_2 the respective numbers of observations at each α with

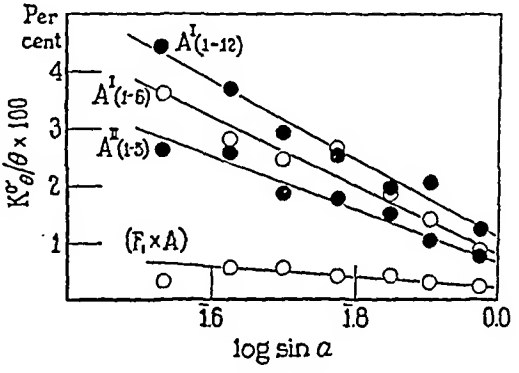


FIG. 4. Data for determining Δ (100 $K\sigma_\theta/\theta$)/ Δ log sin α for various series of observations. See Table III, and text.

each individual (constant at every α for each race). Deviation from the expected ratio would afford measure of unlikeness, as regards variability of the response, in the two races. In other words, we may compare the values of the products

$$\left(\frac{\Delta\ (100\ \text{P.E.}_\theta/M)}{\Delta\ \log\ \sin\ \alpha}\right) \times \left(\sqrt{\frac{n}{N}}\right).$$

In doing so, it has to be remembered that slight errors in fitting may make relatively large differences in the slopes, and thus in the final figures. The slopes (Δ P.E.$_\theta/\theta$)/(Δ log sin α) are obtained from plots (Fig. 4), taking Δ log sin α = 0.4. Table II contains a summary of the available data for races *A*, *K*, F_1 (*K* x *A*), *B*, and *R. rattus*. The num-

ber obtained by this procedure has the significance of a "mean change in the mean root-mean-square deviation from the mean, per individual, as a percentage of the mean, and per unit change of the independent variable." Its use in this form is justified only when the individuals constituting a sample are fairly comparable with one another, but the general method has important possibilities of application to other and quite different cases. The essence of the method consists in the

TABLE II

A measure of the comparative variability of the geotropic response in different lines of young rats is obtained by comparing the values of the products

$$[\Delta\ (100\ \text{P.E.}\theta/\theta)/\ \Delta \log \sin \alpha]\ [\sqrt{n/N}],$$

where α is the slope of the surface, θ is the orientation angle, and P.E.θ (or C.V.θ from weighted averages) is computed (for the *mean*) in the usual way; n = number of observations with each individual, N = number of individuals; n and N are constant, and the *same* individuals are used, at each magnitude of α in any one series. The figures in the last column are taken as direct measures of the fraction of intrinsic, internal, variability of geotropic response which is susceptible to restriction by increasing the geotropic excitation. Discussion in text. See also Table X.

(1) *Race and Series*	(2) N	(3) n	(4) $\sqrt{n/N}$	(5) $\Delta\ 100\ \frac{P.E.\theta}{\theta}\ /\ \Delta \log \sin \alpha$	(6) (4) x (5)
K I	2	20	3.17	1.75	5.55
K III	4	10	1.58	3.80	6.00
K II*	2	15	2.74	0.55	1.50
K + weight	4	10	1.58	1.67	2.64
A I_{1-6}	6	8.3	1.18	2.40	2.83
A I_{7-12}	6	10.0	1.29	1.80	2.32
A I_{1-12}	12	9.2	0.875	2.70	2.36
A II	5	15	1.74	1.80	3.13
F_1 (*K* x *A*)	12	10	0.915	2.10	1.92
(F_1 x *A*)	21	20	0.977	0.36	0.352
B	5	10	1.42	2.67	3.79
R. rattus	5	3	0.74	3.6	2.67

formulation of *varibility as a function of some controlling variable.* Where duplicate series of observations permit direct comparisons, the agreements are clearly as close as can be expected. (It may be mentioned that for the *K* race the "variability number" can also be computed from data on the *speed* of progression (Pincus, 1926–27); it is of the order of magnitude given for angles of orientation (Table II)). And there are equally clear differences between the several groups. To interpret these differences it has to be noticed that (other things equivalent) the slope Δ (100 P.E.$_\theta$/M) / Δ log sin α is an *inverse* measure of the uncontrollable intrinsic general variability of the recorded behavior of the animals in the experimental situation, including errors of recording, but a *direct* measure of the variability of response due to factors susceptible to restriction by increasing the magnitude of the geotropic vector. In this sense, the F_1 (*K* x *A*) individuals are significantly more variable in their geotropic response than their *K* or *A* parents, so far as concerns the operation of influences (affecting the measured responses) which are susceptible to restriction by increasing the geotropic excitation; though, as we shall later be able to demonstrate, the F_1 group should, organically, be in this respect much closer to *A* than to *K*, as it is indeed found to be. We may note that at the time of observation this could not possibly have been foreseen. Although this increased uncontrollable variation in F_1 may be at first sight curious in the genetic sense, it is entirely consistent with the expected effects of hybridization, and may be related in part to the greater weights of the individuals in the hybrids than in the parents, at the time of experimentation.

When the function $\Delta\,\theta\,/\,\Delta\,\alpha$ is the same for two individuals, but θ is higher, consistently, with one than the other, this makes the P.E. of the mean θ larger than it "ought" to be; and it increases that fraction of the total variability of the mean θ's which cannot be proportionately reduced by increasing α. This phenomenon is clearly evident in our detailed records, and is very likely related to the weight of the animal. Hence our comparison with heterosis. This is still further borne out by the data from a populatiou produced in the backcross F_1 x *A* and known to be in this respect heterogeneous (Fig. 4). Again, as we shall subsequently discuss, the residual variability measured in this way and open to influence by increasing sin α, is reduced

by attaching a mass to the animal's body (*cf.* entry "K + weight," in Table II), as should be expected.

For completeness sake, and also because it provides an interesting check upon these contentions from a quite different aspect, we include a series of observations with rats of race K, Series II, already referred to (Crozier and Pincus, 1927–28) as "unsatisfactory" because of technical difficulties. The results are given in Table III, and the computed variation index is included in Table II (K, II*).

Direct observation led to the rejection of this series as relatively nonsignificant, because creeping was more erratic than usual, and in correlation with circumstances clear to the observers at the time (low temperature, mechanical disturbances). In spite of the fact that θ increases so much more rapidly as α increases than in

TABLE III

Orientation Data for Series II, Race K; See text

α	θ	P.E.$_\theta$/θ x 100
degrees	*degrees*	*per cent*
15	26°.7	0.79
20	33°.5	0.81
25	41°.7	0.53
30	51°.1	0.37
35	59°.3	0.25
40	63°.2	0.27
45	66°.5	0.26
50	70°.8	0.18
55	75°.9	0.15
60	79°.3	0.16
65	80°.7	0.32

standard series with this race, Δ (P.E.$_\theta$/θ × 100)/ Δ log sin α is *less* than in series where no persisting sources of irregularity were apparent, and the index of variability employed in Col. 6, Table II, is accordingly much less than in the other series—proportionately less of the total variability is susceptible to geotropic control.

Individuals of the F_1 generation produced by crossing lines K and A were separately investigated, in two groups of 6 each. The F_1 progeny were of course all of the black hooded type (in view of the constitution of K and A). At the time of the tests these animals were 13–14 days old, and weighed 21.93 ± 0.20 gms.; these weights are, as was to be expected, slightly higher than in the parent stocks at the same age

(*cf.* Crozier and Pincus, 1927–28). Any gross influence due merely to increased weight would be expected (*cf.*[1] refs.) to increase θ, at a given slope of surface. But it is clear (*cf.* Table IV; Fig. 5) that the θ's actually found fall definitely within the limits set by the values provided by lines *K* and *A;* therefore, for the moment, this difference in weight can safely be ignored in the further treatment.

The results from these experiments showed that the mean θ's were strictly comparable among the separate individuals. In Table IV

TABLE IV

Mean angles of upward orientation (θ) in two groups, six individuals in each group, of young rats in the F_1 generation of crosses between lines *K* and *A*. The general means of the θ's agree closely with the averages of the means of the two groups, the former being used for subsequent computations. Since the numbers of observations at the several magnitudes of α are not the same, the variability of the measured θ's can be compared in an exact way only through the coefficients of variation of the weighted means, which are therefore given (in the last column, with their probable errors).

α	θ			C.V.$_\theta$
	(i)	(ii)	(average)	
degrees	*degrees*	*degrees*	*degree*	*per cent*
20	46.75	52.19	49.42±1.01	3.05 ±0.42
25	53.48	55.54	54.57±0.86	2.34 ±0.32
30	62.38	59.12	60.39±1.10	2.71 ±0.39
35	64.12	58.37	62.10±0.73	1.75 ±0.24
45	68.82	69.07	68.62±0.58	1.25 ±0.17
55	77.70	75.48	76.55±0.69	1.35 ±0.19
70	82.20	81.03	81.68±0.45	0.818±0.11

the data for the two series are given, together with the general means of the average θ's for the separate individuals, with their probable errors, and the respective indices of variability. In previously recorded series of data[1] we have used experiments in which the numbers of observations were kept the same, thus permitting direct use of P.E.$_\theta$ as a measure of variability. When, as in the present series, the number of observations fluctuates somewhat (though not greatly) among the separate individuals (*viz.*, from 7 to 12) it is necessary to compute the coefficient of variation (Table IV). It was earlier demonstrated[1]

that if the number of individuals be kept *the same* at different magnitudes of α, and the number of readings made with each, the index of variability of θ then declines in a rectilinear fashion as log sin α is in-

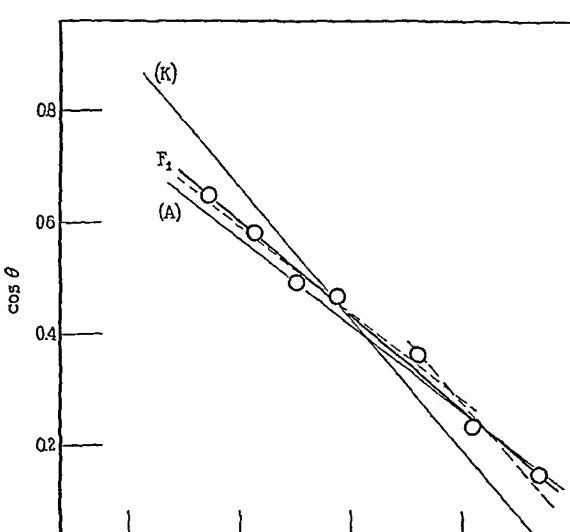


FIG. 5. Mean orientations of 12 individuals produced by crossing $K \times A$. The thin lines on the plot are those fitting the relationship $\Delta \cos \theta / \Delta \sin \alpha = -$ *const.* for the two parental races, transferred from Fig. 1. (In this figure it is assumed that the data can properly be fitted by a straight line. Another and more accurate interpretation (dashed line) is introduced subsequently.)

creased. This relationship, reasons for the existence of which are discussed elsewhere (Crozier and Pincus, 1926–27, *a;* 1927–28), becomes in fact a rather delicate test of the homogeneity of the popu-

lation of individuals employed, as well as of the conditions of the experiments themselves. In the present instance it is apparent that $C.V._{\theta}$, and its probable error, decline in the expected way. It may be noted that for a geometrically quite different case, in which θ is a rectilinear function of *sin* α, not (practically) of log sin α, the variability of θ declines in direct relation to *sin* α, not to log sin α (*cf.* Kropp and Crozier, 1927–28). The validity of this test for homogeneity is perhaps more easily apprehended in the light of the result of its application to a group of young rats similar in a general way but deliberately contrived to be genetically diverse as concerns the elements controlling θ as a function of α; in this case (see p. 78) $C.V._{\theta}$ fails to decline rectilinearly with log sin α, and in fact the mode of its deviation from such a course can be predicted.

The relationship between cos θ and sin α is essentially direct, as shown in Fig. 5, and the slope of the line defined by the relationship is very close to that earlier obtained for the *A* parent (*cf.* Crozier and Pincus, 1927–28). The differences between the mean θ's for F_1 and line *A* are small, although just beyond the ranges of the probable errors, but the fact of the *consistent* difference between the two tends to favor belief in their real separation. It will be noticed that if the connection between θ and α should in fact depend, for lines *K* and *A*, upon a relevant genetic difference between the two, then we might expect that in F_1 the magnitudes of θ found should correspond to those occurring *between* the limits set by the observations with lines *K* and *A* respectively, and not far outside these limits; and if a fairly simple genetic situation should obtain, we might expect the values to be rather near those pertaining to one of the parent lines. These are indeed the facts. At the same time, wide divergence from these expectations would not necessarily preclude genetic analysis; but we appear to have at hand a somewhat fortunate case, in which the absolute magnitudes of the orientation angles and the slope of the line connecting cos θ with sin α are both within the limits set by the parental stocks. At higher values of α, the mean θ's tend to agree rather closely with those established for line *A*, so that the gross *primary* change, in F_1, may be viewed as an alteration of the *slope* of the *cos* θ *vs.* sin α relationship.

These F_1 individuals, which should be a homogeneous group, genetically, and are shown by the statistical test to be a homogeneous group as to their geotropic behavior, were raised to maturity and crossed to lines K and A. Lines K and A had been shown through the earlier work to be in their respective ways also homogeneous, and found to provide consistent values of θ in successive generations and after con-

TABLE V

Mean orientation angles (θ) for upward geotropic progression as determined by the slope of the surface (α), from observations with 22 individuals resulting from back crosses of $F_{1(K \times A)}$ with line K. The mean θ's are computed as averages of the mean angles of orientation gotten for the separate individuals. If the preceding analysis is essentially correct, the individuals procured in this back cross should constitute a population genetically heterogeneous with respect to the elements determining θ as a function of α. We expect then two consequences of this heterogeneity: (1) the smooth connection between $\Delta \cos \theta / \Delta \sin \alpha$ should be noticeably disturbed, although the general character of the connection between θ and log sin α should be more or less the same; and (2) with ($F_1 \times K$) the *variability* of θ should *not* decrease in rectilinear fashion as log sin α increases, but should instead go through a minimum at about $\alpha = 35°$ or slightly above; Fig. 6 shows that this is indeed a fact.

α	$(F_1 \times K)$		$(F_1 \times A)$	
	θ	C.V.$_\theta$	θ	C.V.$_\theta$
deg.	*degrees*	*per cent*	*degrees*	*per cent*
20	52 21±0.40	1.13 ±0.11	52.54±0.99	2.85
25	57.11±0.25	0.637±0 065	57.44±0.85	2.18
30	60.82±0.26	0.638±0.065	60.83±1.14	2.78
35	66.71±0.27	0.596±0.061	64.52±0.73	1.67
45	73.50±0.39	0.786±0.080	70.14±0.27	1.71
55	78.24±0 33	0.63 ±0.064	76.00±0.99	1.93
70	80.90±0.39	0.707±0.072	80.84±0.69	1.28

siderable intervals of time (upwards of 2 years). In the back-cross generation the color and eye-factors were found to segregate in the expected ways, but we are not at the moment concerned with this. From these crosses 21 individuals were taken at random (by litters) in families $F_1 \times A$, and 22 in families $F_1 \times K$, in each case comprising an approximately equal number of males and females. These indi-

viduals were tested separately, employing standard procedure. The results will presently be considered in detail. At the moment we desire merely to consider the *average* results. If our premises are thus far sound we are prepared to encounter the following: (1) the slope of the line of mean θ's should now be consistently nearer to that of the *averages* of the corresponding values for the K and A lines; (2) the cos θ *vs.* sin α relationship, for mean θ's, might be seriously disturbed, or at least definitely modified; (3) the variability of the mean θ's

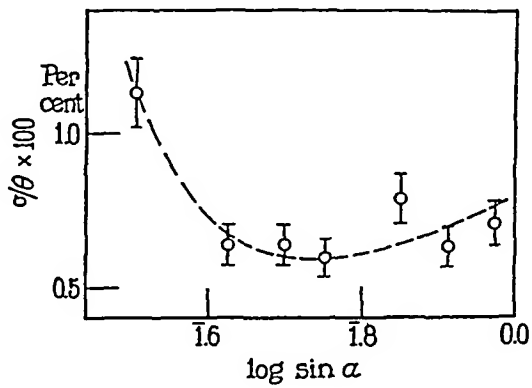


FIG. 6. The variability of θ in a population of young rats deliberately arranged to be gentically heterogeneous fails to decline regularly with increase of *log sin* α. In this case the (22) individuals derived from the backcrossing of F_1 $(K \times A)$ with K were expected to be diverse as regards factors affecting the amount of geotropic orientation; the graphs connecting θ with α for the two parental lines intersect at about $\alpha = 35°$; hence we look here for σ /M to be a minimum at about $\alpha = 35°$, which is the fact, and (*cf.* Fig. 2) we expect the descending limb of the curve to be the steeper.

$(F_1 \times K)$ should no longer be found to decline directly with increasing log sin α, but should pass through a minimum at $\alpha = 35° - 40°$, although the rising branch of the curve ($\alpha > 40°$) should not be so distinct as the descending arm ($\alpha < 40°$). These expectations arise from the fact that if segregation of factors concerned with the manifestation of θ as a function of α does indeed occur, then the mean of this $(F_1 \times K)$ population should return toward that of the two P lines, the θ-graphs for which cross at about $\alpha = 35°$ (Fig. 1).

The significant test is made by contrasting the behavior of the $F_1 \times K$ individuals with that in line K, since the F_1 data show at least *relatively* complete dominance of factors in the A line (*cf.* Fig. 5). Table V contains the necessary data. It is seen that C. V.$_\theta$ does indeed behave in the expected way (Figs. 6, 7), while it is clear (*a*) that the mean θ's are about midway toward those exhibited by rats of line K, and (*b*) that the cos θ *vs.* sin α curve does appear seriously affected.

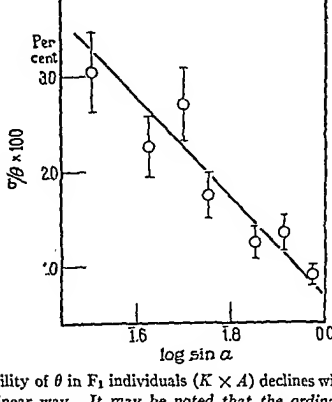


FIG. 7. Variability of θ in F_1 individuals ($K \times A$) declines with *log sin* α in an essentially rectilinear way. It may be noted that the ordinate scale is quite large; σ /M $\times$ 100 is plotted as a bar, the height being = 2 P.E. of the ordinate.

The θ-curve *slope* is more interesting than the absolute magnitudes of the θ's, as we will shortly have occasion to see. The very significant behavior of C.V.$_\theta$ we have a right to regard as a direct justification of the reasoning which initially led us to the investigation of genetically stabilized lines. Since the absolute differences which concern us are small, it is of interest to demonstrate that the rate of decline of variability of the mean θ's for the individuals from the opposite backcross, ($F_1 \times A$), is actually *less* than with the individuals from ($F_1 \times K$), as it should be if the premises already discussed are efficient. Table

V contains these data also. It is apparent that the variability declines with increasing α, as we have a right to foresee, but that there is no real minimum (C.V.$_\theta$ has been corrected for the number of observations at each slope). It is necessary to remember again that variations in weights of individuals may be significant here, or other causes leading to change of θ without change of $\Delta\,\theta\,/\,\Delta \log \sin \alpha$; these influences will affect the absolute magnitudes of C.V.$_\theta$ as computed.

ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. II

By W. J. CROZIER and G. PINCUS*

(*From the Laboratory of General Physiology, Harvard University, Cambridge*)

(Accepted for publication, April 10, 1929)

IV

It has been pointed out that for these cases θ (mean or median) is very nearly related to *log sin* α, in such a way that

$$\Delta\theta/\Delta \log \sin \alpha = \text{"}const.\text{"} \quad (2)$$

In every series of data, however (Crozier, 1928; *cf.* Crozier and Pincus, 1927–28; Crozier and Oxnard, 1927–28; Keeler, 1927–28), the measurements really fall upon a sigmoid curve (*cf.* Fig. 8). The constant recurrence of this form of the curve forces its recognition, although we were at first content to use the approximate logarithmic relationship because it is adequate for some purposes (Crozier and Pincus, 1926–27, *b*). The two end regions of these curves are hard to establish precisely, owing to increasing variability of movement at low values of α and to difficulties of progression at very high inclinations ($> \alpha = 70°$). But there is no reason to question the general form of the curves. If we assume an array of peripheral receptors which may be stimulated in increased number (or with greater frequency) as the labor of upward progression is increased (*i.e.*, as α is made higher,) then, on the basic assumption that upward orientation ceases with sensible equivalence of excitation on the two sides of the body, the angle Θ must be taken as a direct measure of the mean total number of activated sense organs per unit of time. "Sensible equality of excitation" means that not more than one "group" of receptors is stimulated, on the average, on one side in excess of those stimulated on the other side. But the total number of activated receptors (pro-

* National Research Council Fellow.

prioreceptive tension-receptors, on this view,) will depend not only on the magnitude of the exciting force (here proportional to *sin* α) but also on the distribution of excitation-thresholds among them. The relation of this conception to Hecht's (1923–1928) analysis of retinal excitation should be clear, although the two are by no means identical,

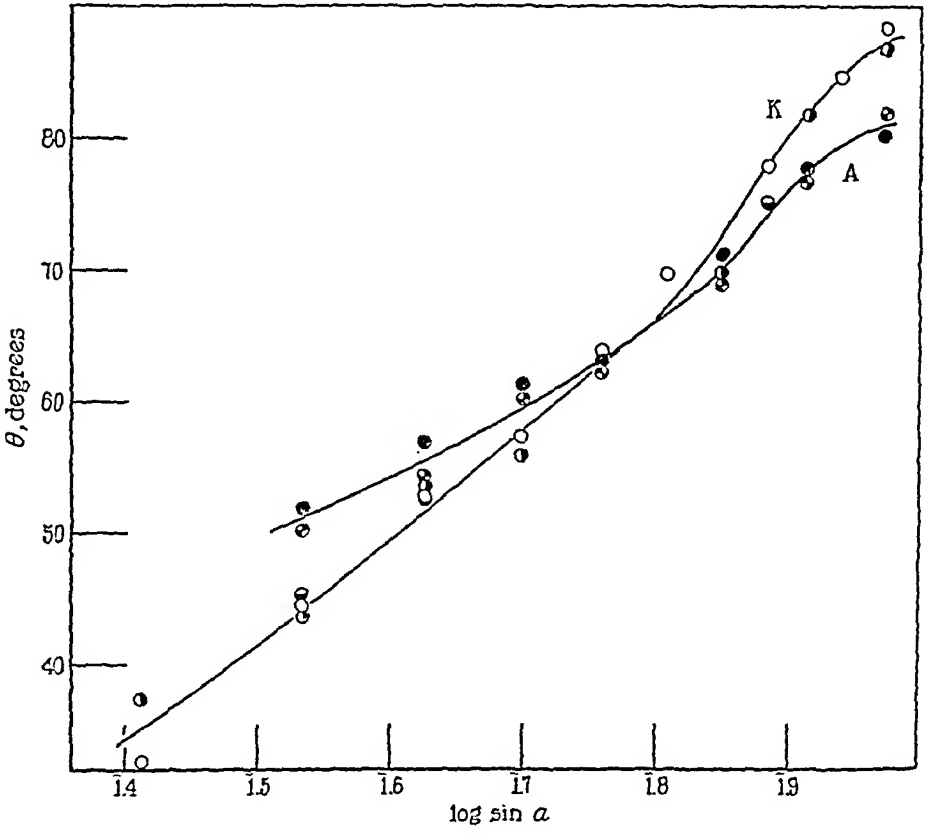


FIG. 8. θ as a function of log sin α, for lines *K* and *A*.

as we will find reason to see. The ratio d θ/d *log sin* α (that is, the slope of the curve connecting θ and *log sin* α) gives the rate at which additional excitation is involved as *sin* α increases. Unquestionably, the matter of *frequency* of excitation, as related, mainly, to the speed of creeping (*cf.* Pincus, 1926–27) and thus to the frequency of movement of the appendages, introduces complications. Tension-excitation depends on *change* of tension (*cf.* Adrian and Zottermann, 1926;

Cooper and Creed, 1927; Adrian, 1928). We are justified in exploring the consequences of simplifying assumptions, provided we do not forget their existence, and we shall accordingly assume that while the effective gravitational pull is proportional to *sin* α, the excitation of a single receptor unit over an interval of time during progression depends upon *log sin* α. It is significant here that the speed of progression, approximately determined by the frequency of stepping, is known to be directly proportional to *log sin* α (Pincus, 1926–27); more accurately, in the data at our disposal, θ and speed of progression are directly proportional.

For a given Θ, there is equivalence of numbers of tension-receptor sense organs activated on the two sides of the body, and constant *frequency* of excitation over gross intervals of time. Then θ is a direct measure of the total excitation experienced. It is on this basis that the relation $\Delta \cos \theta / \Delta \sin \alpha = -$ *const.* is obtained.[1] When α is varied, the speed of creeping changes, also θ. We can regard this as signifying that both the total number of receptors and the frequency of excitation alter with α, and so with θ. Hence, θ is such a function of α that, as α varies, it in some fashion includes both elements in the totality of excitation over a gross unit of time. Assuming that for any small increase, ΔE, in total excitation per unit time, we have $\Delta E = K_1 (\Delta N) (\Delta F)$, where N signifies number of receptors, F frequency of excitation through changes in the positions of the limbs during progression, then since $\Delta F \propto$ Speed of progression, at least approximately, and since Δ Speed/Δ *log sin* α is sensibly constant,

$$\Delta E = K_2 (\Delta N) (\Delta \log \sin \alpha).$$

But our assumption must be that Θ and E are equivalent,

$$\therefore \Delta \theta = K_3 (\Delta N) (\Delta \log \sin \alpha)$$

and $\dfrac{\Delta \theta}{\Delta \log \sin \alpha} = K_3 (\Delta N)$. On this basis, plotting $\Delta \theta / \Delta \log \sin \alpha$ against *sin* α should show how increasing α brings new sense organs into play according to the distribution of effective tension-thresholds among them.

In this connection it may be remarked that lowering the temperature decreases speed of movement, and θ, at given α; while attaching (moderate) added loads increases both. It should be clear that the *logarithmic* factor in these computa-

tions is not to be considered as other than a conveniently adequate relationship; and with respect to any tendency which may exist to regard it as signifying "Weber's Law" we may point out that here the approximately logarithmic relation holds over the *whole working range*, not to its mid-portion (*cf.* Hecht, 1923–24).

The phenomena of "post contraction" in the human arm show how one aspect of the direct proportionality of continued excitation to motor effect may be tested

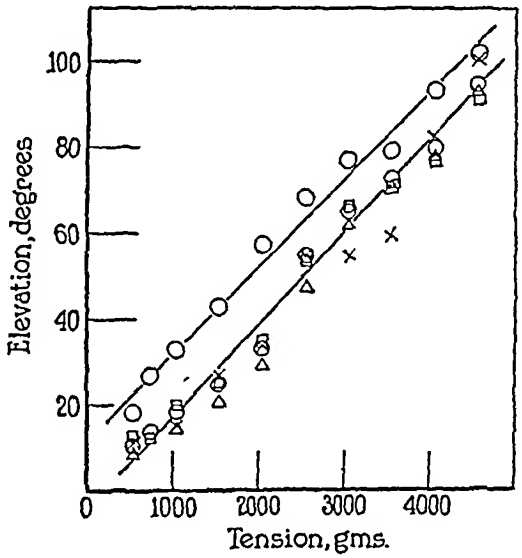


FIG. 9. Data on the angular elevation of the human arm (post contraction), released after withstanding various tensions (from: Allen and O'Donoghue, 1927). The uppermost set pertains to one subject, the lower sets to another. See text.

objectively. Allen and O'Donoghue (1927) measured the post contractional angular displacement of the arm after submission to known tensions. Although they analyze their results in a quite different way, it is clear from the data that the angular displacement, which (rather than the work done in elevating the released arm!) may be taken as a measure of the "intensity" and therefore of the *persistence* of the initially excited flow of proprioceptively originating impulses, is in fact directly proportional to the exciting force (cf. Fig. 9). In many other instances the attempt to find "logarithmic" relationships is equally out of focus.[4]

---

[4] We may anticipate certain matters which it is expected to develop elsewhere, to the extent of pointing out that the nature of the limitation of geotropic orientation as here considered is essentially identical with the ancient problem of lifted weights in psychology. It can be stated that analysis of the existing data appears possible, without appeal to "units of sensation" but in terms of numbers of affected tension-receptors, by a method analogous to that employed in the present paper.

It will have been noticed that for the distributions of measurements given in Figs. 8, 12, *etc.*, smooth curves have been drawn. This is entirely justified under the conditions, since each plotted point is subject to a certain (measured) precision, which is taken into account in drawing the smoothest permissible curve. It may well be that if greater refinement of observation were possible, discontinuities of an abrupt sort would become apparent, but if the notions employed in

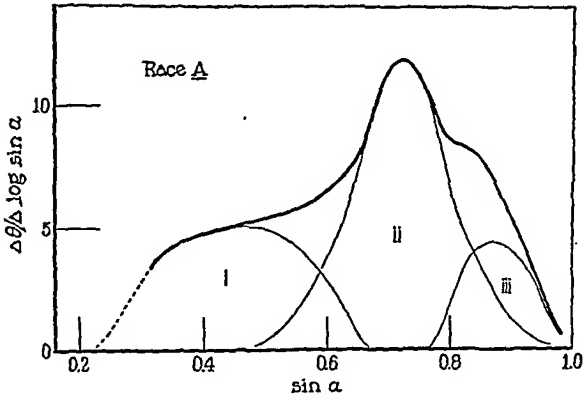


FIG. 10. In arbitrary units the differential $\Delta\theta/\Delta \log \sin \alpha$, obtained graphically, from the data for race *K* (*cf.* Crozier and Pincus, 1927–28; and Fig. 8 of the present paper) is plotted against $\sin \alpha$. The resulting curve (full line) is analyzable into a central symmetrical distribution curve, and the residues at either side provide by difference the right- and left-hand groupings. Within reasonable limits, I, II, and III are each symmetrical.

this paper are approximately correct there seems, in the case of this material, no opportunity to achieve such precision; nor is there any likelihood that it would appreciably affect the main results of the analysis even if secured.

When $\Delta\ \theta/\Delta \log \sin \alpha$, obtained by measuring the slopes of the fitted curves (Fig. 8) (by the mirror method, for example), is plotted as a function of $\sin \alpha$, the differential curves appear as in Figs. 10

and 11. These graphs clearly are not simple. But for the fact that suggestive comparisons may be made among the curves available for the several races, it might be unprofitable to attempt to use them. As they stand, the differential curves, necessarily incomplete at the low-α end, each appear to be composed of 3 almost symmetrical population curves. This can be taken to signify, if further tests support the

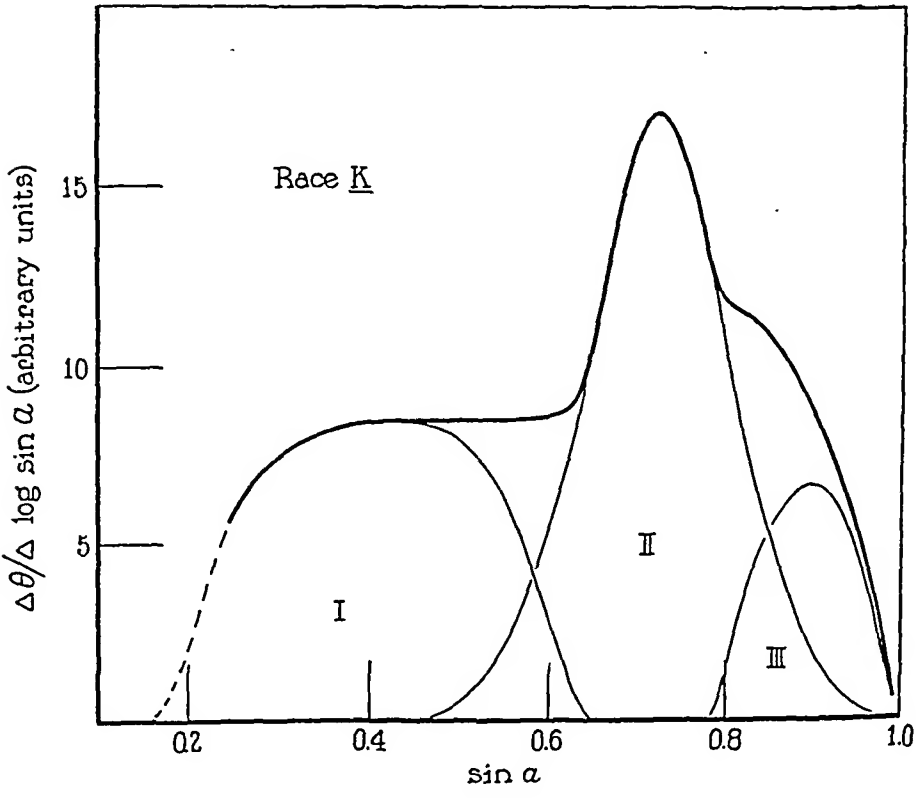


FIG. 11. An analysis of $\Delta\theta/\Delta$ *log sin* α *vs sin* α for race *A* shows essentially the same condition as in the case of race *K*, except that *i*, *ii*, *iii*, are each smaller than the corresponding elements in *K;* and the positions of the maxima are *slightly* different.

notion, that there are involved 3 large groups of receptors, of successively higher mean excitation thresholds, the implication of the members of these overlapping groups in the determination of the angle θ proceeding additively as *sin* α is made larger. The three groups may be labelled I, II, III in race *K*, i, ii, iii in race *A*. Whether these "groups" are, as such, distinct entities, or that merely the mechanics

of the involvement of receptors in excitation is diversified in this way, we are not at the moment called upon to answer.

The triplicate character of these curves reappears in the analysis of the data pertaining to race *B* (Fig. 12, 13). When the three races are compared the differences between them are seen to be of the following kinds. Disregarding the magnitudes of θ at the lowest workable slopes, and paying attention only to Figs. 10, 11, 13, races *A* and *K*, which chiefly concern us, show but slight differences between the mag-

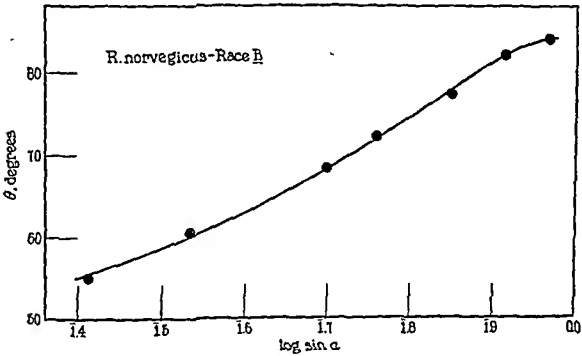


FIG. 12. θ as a function of *log sin* α, for race *B*.

nitudes of *sin* α at which the peaks of our separate distribution curves appear. The only real differences concern the sizes of the three sub-curves into which each curve has been analyzed. The central curve for *A*, in fact, is drawn by multiplying the corresponding ordinates in the *K* curve by a constant. Within the limits which are completely negligible in view of the errors of the graphical procedure, the same is true of the curves for *i* and *iii*. The *A* individuals then differ from the *K* in the magnitudes of the ordinate units in which $\Delta\ \theta/\Delta\ log\ sin\ \alpha$ is to be expressed. Essential differences between *A* and *K* should be revealed by reducing the curves for the two to a common basis, involving (1) a threshold θ, (2) a constant for $\Delta\ \theta/\Delta\ log\ sin\ \alpha$. The analysis

of the θ vs. *log sin* α curve for race *B* (Fig. 13) shows groups 1, 2, 3 again easily recognizable, but to be in each case small. The similarity between *A* and *B* we have already seen to be chiefly a matter of the geotropic threshold (Fig. 1, 8), rather than of the function $\Delta\theta/\Delta\alpha$. Hence if we adjust the ordinate scale to give equivalence in rise of θ over the working range of geotropic excitation the curves for *A* and *B* should be made to coincide, at least approximately. This might be

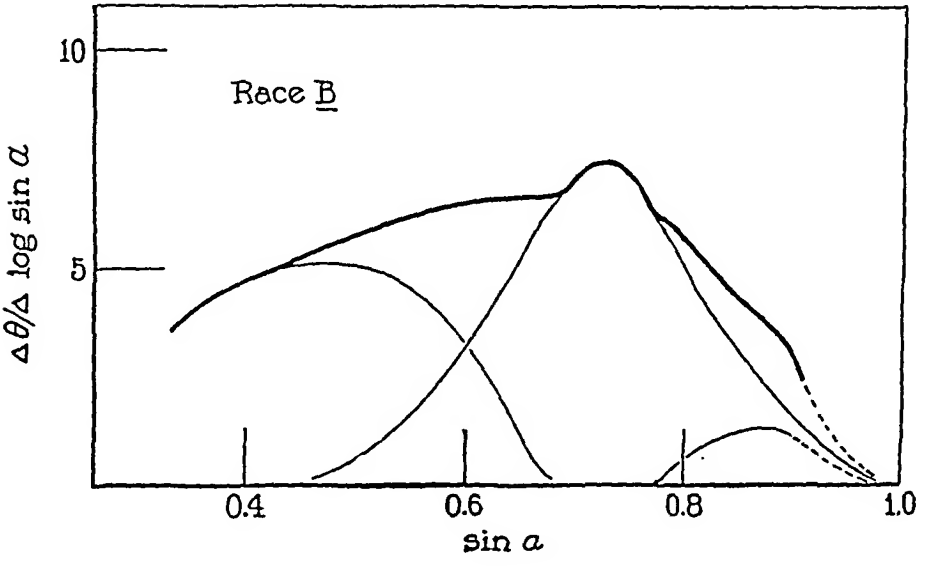


FIG. 13. Plot of $\Delta\theta/\Delta$ *log sin* α for race *B*. The modes of the 3 "groups" are at practically the same points (*sin* α) as with *K* and *A* races, but the "groups" are each smaller. (The extreme right hand end of this curve is uncertain; see text, and Fig. 2.)

regarded as the effect of a difference in a central nervous threshold determining the liminal difference in excitation on the two sides of the body. No such simple adjustment can be made with the data from *K* and *A*, however, which can abolish the differences revealed by Figs. 10 and 11.

Relations of the same sort appear in the case of young mice (*cf.* Crozier and Oxnard, 1927–28). The orientation of slugs (Wolf, 1926–27), and of other forms, involves different considerations, which cannot be gone into here.

The lowermost "group" is incompletely represented, since $\Delta\theta/\Delta$ *log sin* α cannot be shown to drop to zero within the range of values of α open to reliable test. But if we assume it to be a really symmetrical curve (*i.e.*, *I* and *i*) we can arrive at a sort of approximation of the lowest slope effective in evoking geotropic creeping. For the *K* race, this is (Fig. 10) at about 9°; for the *A*, about 5°; and for *B*, about 6°.

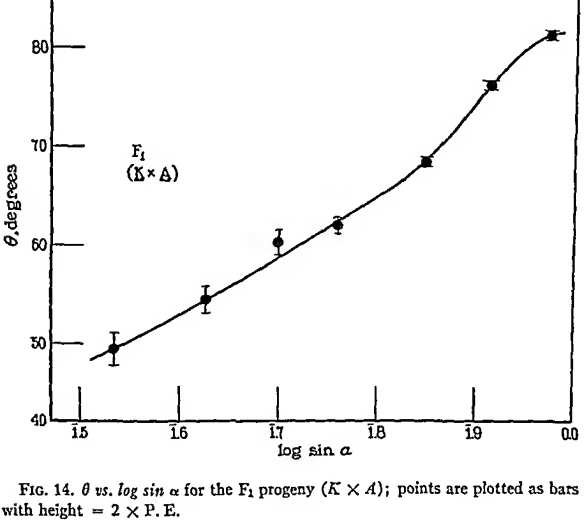


FIG. 14. θ *vs. log sin* α for the F_1 progeny ($K \times A$); points are plotted as bars with height = 2 × P. E.

These values are "ideal" however, and except in the case of *K* do not correspond to the indications of the *cos* θ *vs. sin* α plots; in this respect races *A* and *B* must be regarded as having certain of the receptors concerned in the determination of θ already activated when the individuals of the age we have employed are creeping on the flat (*cf.* Fig. 1).

We might proceed in several ways to test the adequacy of this representation. It may be possible, for example, to modify the

curves in Figs. 10, 11, 13 when the rats carry additional loads (attached masses). One might obtain in this way evidence as to the possible anatomical separateness of the three presumptive receptor groups. For the time being, however, we confine attention to a genetic test.

In Fig. 14 the connection between θ and *log sin* α is plotted for the F_1 individuals from the cross $K \times A$. It may be mentioned that the

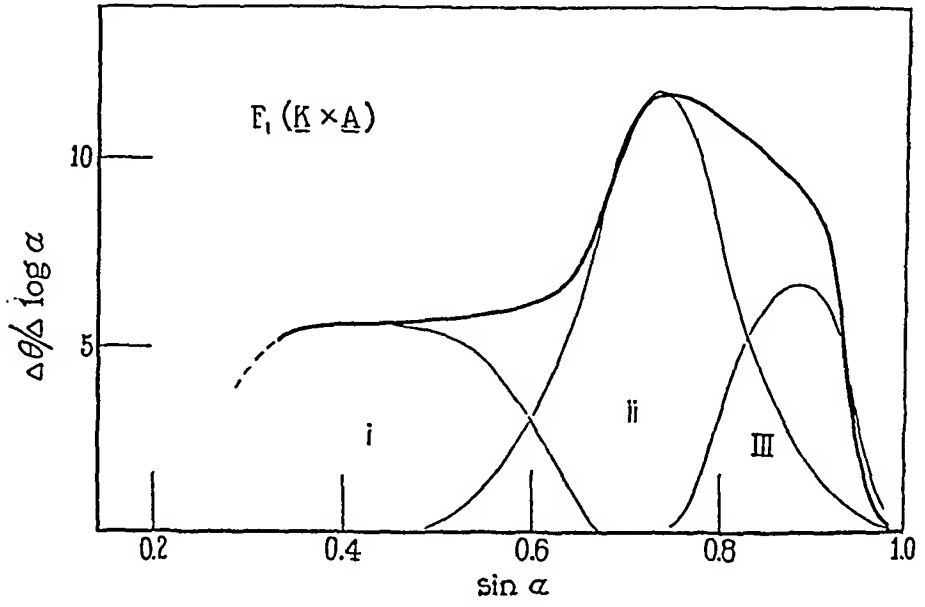


Fig. 15. $\Delta\theta/\Delta \log \sin \alpha$ (full line) is drawn for the F_1 individuals from $K \times A$. The three regions distinguished in the curves for the original races are again clear. The central (dashed) distribution curve is traced from that found with the *A* race (Fig. 11); the left hand curve is also traced from that for this race (*A*); the right-hand distribution is that for race *K*; except for the extreme right hand end of the graph, which is in any case uncertain, the parental resolution curves *iA*, *iiA*, *IIIK* give an entirely adequate picture of the state of affairs in the progeny; see text.

respective curves were drawn in their final forms before this mode of treating them was contemplated. It appears (Fig. 15) that the F_1 individuals resemble the *A* race in that "groups" 1 and 2 are closely enough alike to be taken as identical (*i.e.*, they are *i* and *ii*), while the area and form of "group" 3 is here an exact duplication of that in race *K*; in fact, the constituent curves are traced from the respective graphs already given. The only differences lie in the fact that *i* in

F_1 is slightly larger than in *A*, but obviously not like *I* in *K*, so that its curve must be slightly expanded (as in Fig. 11, dashed line); and there are evident slight shifts in the positions of the maxima—that for ii being in F_1 pushed 0.06 unit to the right, and for III 0.08 unit to the left (this makes III assume, as regards its maximum, exactly the *A* position, i the *K* position, as seen in Figs. 7, 8).

The naïve interpretation of these effects might be to regard the conditions producing small *i* and *ii* groups to be dominant over the corresponding large I and II; and reciprocally as regards III and iii. *If the developmental bases for these differences are distinct and independent*, more critical examination of the situation should be possible with the help of tests for segregation of their genetic correlates.

V

Individuals from the F_1 generation of ($K \times A$), already used for the orientation experiments, were raised to maturity and back-crossed to the *K* and to the *A* lines. If the suspicion be justified that the three "groups of sense organs" correspond to genetically independent entities, a different outcome is of course to be looked for in each back-cross. The comparison of Figs. 10, 11, 13, 15 suggests that the groups 1, 2, 3 may be in some fashion essentially independent. We then expect the following relationships to appear in the two back-crosses. In ($F_1 \times A$) we expect the individuals to be all very nearly alike, but, given adequate measurements, they should be separable into two classes upon the basis of a difference in size of group *3*; for, if the indications of dominance already seen are lived-up-to the progeny in this back-cross should obviously be of two sorts, phenotypically, one half of them (ideally) showing a large group 3, *i.e.*, *III*, the other half showing *iii*. In the other back-cross, ($F_1 \times K$), we should expect the third group to be essentially alike in all the individuals, but there should be *four* classes as regards the exhibition of large and small curves for groups 1 and 2.

The kinds of difference thus predicted impose a radical test of the antecedent analysis. There is required the establishment of a reaction curve for each individual separately, the grouping of these curves upon an objective basis of the differences they may exhibit, a statistical justification of such groupings, and the examination of the differen-

tial curves which eventuate. We may expect that differences among the weights of the cross-bred individuals may introduce complications, so that this additional point must be examined experimentally.

Four litters were used in the backcross $F_1 \times K$. These animals were born within two weeks of each other. Three litters were produced by mating F_1 females to 60th generation King albino males, the fourth from a mating of a 60th generation King albino female to an F_1 male. Four animals (nos. 19 to 22) were put to nurse on a *B* family female. No influence due to the mother was noticeable although the *B* line geotropic reaction is obviously different from that of lines *A*, *K* or F_1. Similarly 6 animals of one of the three $F_1 \times A$ litters tested were nursed by a *B* line female, with no apparent influence on the geotropic response due to the mother. The three $F_1 \times A$ litters were also born within two weeks of each other, and two months after the $F_1 \times K$ litters. They were all sired by the same *A* line male and the mothers were F_1 females. All the F_1 animals used in these crosses were previously tested for their geotropic reaction. Where the litter size was greater than six the litters were split and part put to a foster-mother. Thus from two to six animals were nursed by a single female, but never more.

The individuals tested in the back-cross ($F_1 \times A$) were 21 in number, 3 litters. Each animal was tested for geotropic orientation, under standard conditions, about twenty readings being secured at each of 7 inclinations. The same creeping surface was used throughout. The mean θ's are collected in Table VI, where the individuals are grouped in a manner to be discussed shortly.

Although the numbers of rats are small, there are several independent tests which may be applied and which give concordant results concerning the reality of the differences detected in comparing these individuals. We have seen reason to expect very slight if indeed any differences in the curves at low values of α, since we would look for the receptor groups 1 and 2 (*i.e.*, *i*, and *ii*) to be identical throughout. But, from the results of experiments with attached weights[1] (*cf.* later), this expectation might be upset if the weight of the young rat is genetically modified, either as to amount or arrangement, in such a way as to act differentially upon our assumed groups of receptors. Table VII shows that there is no correlation, however, between the total

TABLE VI

Average orientation angles secured with 21 individuals (3 litters) in the back cross generation ($F_1 \times A$); separated into two groups on the basis of $\Delta\ \theta$ in the range $\alpha = 45° - 70°$; analysis in Figs. 17 and 18. In this and in several succeeding Tables blank entries signify that for one reason or another no observations were made. In the column headed "*color*", *b* signifies *black*, *y* signifies *yellow*.

($F_1 \times A$) *Group 1 (III)*

No.	Wt., *gms.*	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
26	27.0	b	53.44	59 75	61.56	64.20	71 70	74.79	80.71
28	24.5	y	51.63	56.80	60.80	65.44	73.80	75.75	82.19
30	19.0	b	52 60	—	57.20	61.90	72.40	75.44	82.27
33	18.0	y	50 67	61 50	64 00	63.00	71.10	75.77	82.00
36	24.0	b	51.93	56 47	64.40	66 50	69.40	74.00	80.07
37	25.0	y	51.53	55 25	58.86	64 23	70 27	73.67	78.76
40	25.0	b	49.07	55.06	59 36	61.40	70 00	73.86	80.50
41	24.5	y	51.06	56 75	62 21	65 53	70 80	77 93	81.20
42	24.5	b	52 44	57.87	61.59	66 29	69.56	73.44	79.71
43	28.5	b	52.95	56.50	63 73	65 53	71 40	74.43	80 33
θ *mean* =			51.63	57.33	59.37	64.40	71.04	74.91	80.77

($F_1 \times A$) *Group 2 (iii)*

No.	Wt., *gms.*	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
23	25.5	y	51 89	59.15	63 30	67 10	74 60	78.20	83.27
24	24.5	b	51.65	58.37	59 53	64.53	73.30	77 47	81.53
25	26.0	b	51 37	55 53	59.82	63.13	70 50	78 93	80.29
27	25 0	y	54 00	56 35	62 15	65.63	72 00	76.00	79.75
29	22.5	b	53 07	59.60	63 00	63.40	74.40	75.50	80 68
31	17.0	y	54 09	—	62 00	61 20	74.40	77.87	82.47
32	18 5	b	52 35	—	59 81	61.69	74 10	75 86	79.31
34	17 5	y	51.67	—	58.29	64.27	74 50	78.50	82.00
35	19.0	b	54 67	—	57.64	63.90	74.10	78.00	81.76
38	24 0	y	51.79	57.00	62.38	68.07	69.75	75.44	79.00
39	24.5	y	52.53	56.12	59.87	64.06	69.93	75.20	79.81
θ *mean* =			52.44	57.44	61.02	64.31	72.75	76.90	80.81

weight of individual and the mean θ at $\alpha = 20°$, nor at $\alpha = 70°$. This influence of weight we may then ignore, at least as to any *gross* manifestation (as already noted with the F_1 generation). We can

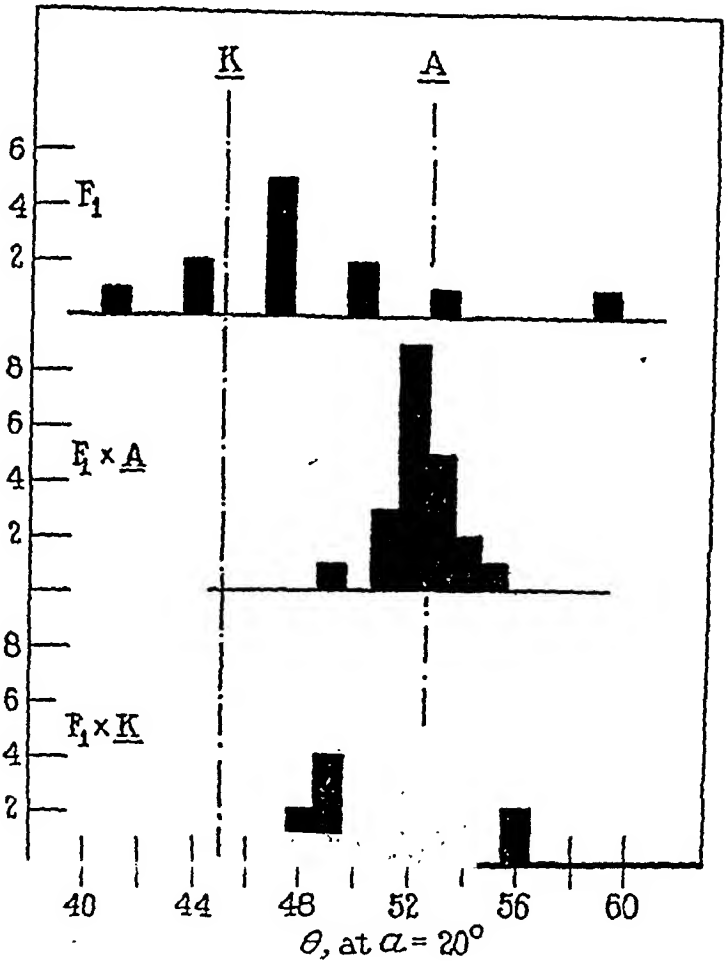


FIG. 16. Frequency distributions of mean θ at $\alpha = 20°$, for F_1, $(F_1 \times A)$, $(F_1 \times K$ populations; mean values of $\theta_{20°}$ for K and A lines are added.

therefore afford to look for a significant shift of θ at $\alpha = 20°$ in com paring the orientations of individuals produced in the two back-crosse It is to be remembered that at this low inclination the θ measuremen are the most variable; so that we might not be surprised to find on blurred indications of the relationships sought. The experiment

facts, however, are detailed in Fig. 16. It is clear that the F_1 individuals show modal θ's between those already gotten with the K and A races, and that with (F_1 x A) the mode moves still further toward the A position—*becomes in fact identical with it.* With (F_1 x K) there is some definite indication of two types of θ, one near the F_1 position—that is, shifted toward K,—while the majority cluster about the θ characteristic of the A race. We shall find independent reason for regarding these distributions as reasonable.

TABLE VII

Distribution of mean θ for each individual at $\alpha = 20°$, as function of weight of animal; ($F_1 \times A$), and in italics ($F_1 \times K$). Showing no correlation in either case.

θ, degrees	Wt., gms.	17.5	19.5	21.5	23.5	25.5	27.5	29.5
	46.5	*1*						
	48.5		*2*	*3*	*1*	1 *1*	*1*	
	50.5	1 *1*	*3*	*1*	3	2		
	52.5	1	2 *3*	*4*		6	1 *1*	1 *1*
	54.5	1	1 *2*	*2*		1 *1*		*1*
	56.5	*1*	*1*		*1*			

The mean θ's at lower slopes for (F_1 x A) individuals are obviously consistent throughout. At the uppermost end of the curve, however, this is no longer true. Careful consideration of the curves for the separate individual shows that they fall into two classes which can be distinguished reasonably well. Up to $\alpha = 55°$ the θ's for both classes show astonishing agreements with the means already established for the A race[1] (*cf.* Table 1). The fact that the agreements are in this respect better than with the F_1 population may be accidental, or due to the genetic reshuffling of unrecognized influences affecting orientation. But above $\alpha = 45°$ the two classes of cases recognized in Table

VI are to be separated. In one of these classes the determination at $\alpha = 55°$ is consistently higher (as in the F_1 population) than in A, while the figures for the other class agree better with those for A. The curves for the mean θ's in the respective classes are given in Fig. 17. There is no real correlation between weight of individual and this grouping, though the iii rats average a little less than the III array. These differences are so slight that in spite of their complete consist-

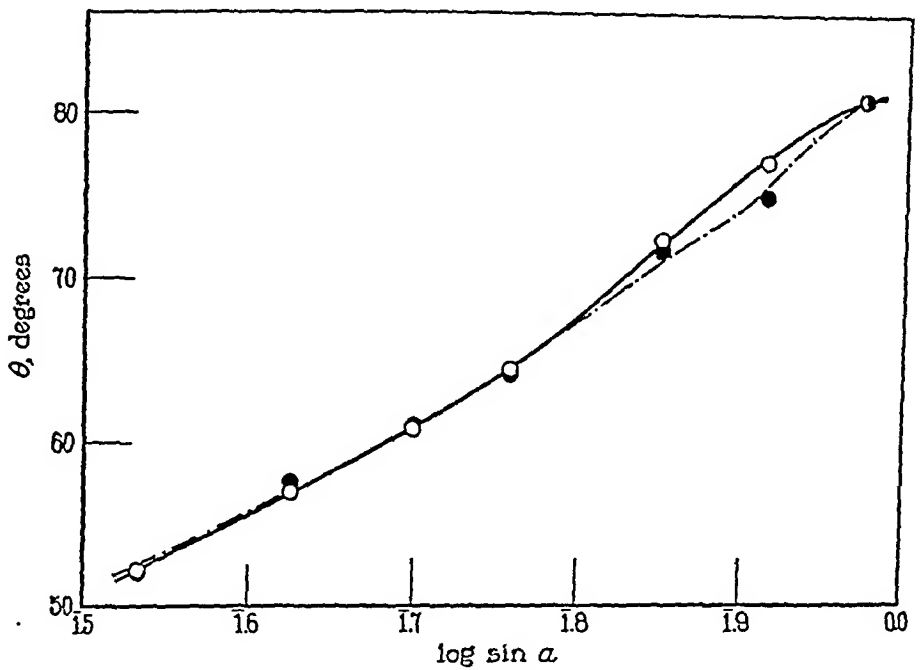


FIG. 17. Orientation-angles (θ, mean) for two groups of individuals (Table VI) distinguished in the back-cross progeny ($F_1 \times A$); in one group (●), the effect labelled III (*cf.* Figs. 10, 11, 13) is apparent, and contrasts with iii in the other group; the only detectable difference is in the region $\alpha = 50°$ to $\alpha = 70°$.

ency with the forecast of the analysis we do not feel that any great significance can be attached to them alone. Their real meaning is that the mere crossing of F_1 and A individuals, found to be closely similar in geotropic behavior, does not give rise to new and unforseen differences among the progeny produced. This *negative* evidence is none the less powerful, and supports the evidence provided by the forms of the curves in Figs. 17, 18. The relations between *cos* θ and

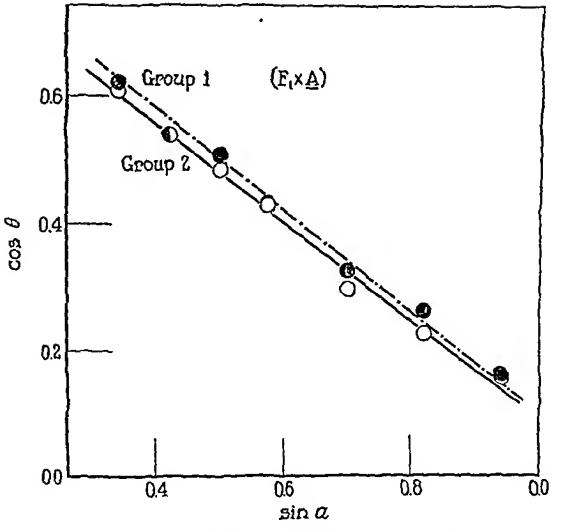


FIG. 18. *Cos* θ vs. *sin* α for the two groups of individuals in the families ($F_1 \times A$). For one of these, the line should be similar to that for F_1; for the other, it should be similar to that found for the A grandparents. The group of composition (i, ii, iii) give a slope $\Delta \cos\theta/\Delta \sin\alpha = 7.8$, with $\Delta \sin\alpha$ taken = 0.4, while for the A line the slope is 7.8 (*cf.* Fig. 1). [The fact that the position of the line is slightly lower on the *cos* θ axis is discussed subsequently in the text; this effect causes the θ — *log sin* α plot (Fig. 17) to be as it were rotated about a mid-point (by comparison with that for A), and thus to signify a broadening of the distribution curves for thresholds of our groups of sense organs.] The group of individuals presumptively (i, ii, III) in constitution should on the present coördinates show a break in the graph, with increased slope at the high α-end. The best fitting straight line should have a slope similar to that already gotten for the similarly constituted F_1 (Fig. 5); it is actually = 8.0 units, as compared with 8.4 units for F_1.

sin α for the two groups of individuals are given in Fig. 18. (At a later point, the variability of mean θ is also discussed). It should be mentioned that sundry random groupings of individuals (Table VI) have been tested, and that in no case do the regularities detected in Fig. 17 any longer appear.

It will be noticed that where we expected large and small examples of "group 3" in the ratio 1:1, we have in Table VI actually 11 of one kind and 10 of the other. This ratio is better than would be expected to occur often in a sample of this size. Consequently it is of interest to find that, from the color-factor constitutions of races *K* and *A*, we look in the progeny of F_1 x *A* for *black* and *yellow* offspring in the ratio 1:1; we have in the litters used for the geotropism tests 10 yellow and 11 black. There is no definite association of our *III* and *iii* forms observable with color (4 were *III*, yellow; 6, *III* black; 6, *iii* yellow; 5, *iii* black). The factual segregation of the color-determining genes, of which the genetic behavior is sufficiently well-known, proves that no untoward selective influence dictated our choice of individuals for testing. It seems to us worth stressing the technical advantage, and the significance, of such self contained checks on the composition of the populations used in the tests, especially when the numbers available cannot be made large.

The results in the other back-cross are expected to present a more complex picture. We look for all individuals to be alike as to "group" III; but, if free assortment of whatever it be that determines the I, i; II, ii association is to take place, we look for *four* groups of individuals phenotypically separable, showing all possible combinations of the "large" and "small" groups in positions 1 and 2. Since it is physically out of the question to manipulate *large* numbers of progeny and yet to keep all necessary conditions uniform, and since our primary aim was to test the nature of differences between our original formulae for the *K* and *A* lines, *any* evidence of segregation is to be looked upon as favorable to the inquiry.

From the indications given by the distributions of θ at 20° (Fig. 16) we have already seen reason to expect in (F_1 x *K*) *two* groups of individuals, separable at low values of α. More complete separation is obtainable in two other ways. With sufficiently reliable measurements we might analyze the θ-log sin α curve for each individual, grouping

TABLE VIII

Individuals produced in the backcross ($F_1 \times K$) are empirically separable, as regards $\Delta\theta/\Delta\alpha$, into four distinct groups, as discussed in the text; *bl.h.* = *black hooded*, *alb.* = *albino*.

($F_1 \times K$) Group 1 (I, II, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
4	21 5	*bl.h.*	49 06	56.32	60.88	63 63	79 00	80.38	84 68
5	19 5	*bl.h.*	47.50	57 29	60 36	61.08	76.29	81.00	82.71
8	20 5	*alb.*	50.52	53.04	59.45	68.44	71.17	80.84	83.59
11	19.0	*alb.*	48.88	53.25	58 90	68.99	74.63	82.53	84.74
19	20.5	*alb.*	49.23	56.23	58 41	68.95	77.94	75.50	86.67
Mean =	20.2		49.23	54.63	59.48	66.94	74.98	80.51	84.53

Group 2 (i, ii, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
1	22.0	*alb.*	56.13	61.15	63.17	64 33	74.07	74.83	81.55
9	20.5	*alb.*	52 75	53.04	59.45	68.44	71.17	80.84	83.59
10	19.5	*alb.*	53.76	56.48	60.41	67.30	72.54	76.32	77.05
12	20.5	*bl.h.*	53.18	59.58	63.08	67.41	70.48	77.76	83.57
13	19.3	*bl.h.*	51.79	57.83	61.59	65.36	70.21	76.11	82.29
21	17 5	*bl.h.*	56.52	58.93	62 47	66 50	76.80	77.44	81.90
22	20.4	*alb.*	54.63	57 43	61 06	67 38	74 61	81.05	81.57
Mean =	20.0		53.66	57.40	61.44	66.84	72.21	77.62	81.64

($F_1 \times K$) Group 3 (I, ii, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
3	21 2	*bl.h.*	51.13	56 58	61.58	56.67	72.46	73.50	78 09
6	20.0	*alb.*	52.13	56.73	62 59	67.25	73.31	80.57	83.65
7	17.0	*alb.*	47.96	55 92	58.82	69.58	68.89	78.17	79.04
15	30 0	*alb.*	51 96	57 00	61.17	66.11	71.55	77.63	83.88
18	28.0	*alb.*	49.11	56 86	57.68	65.44	75.94	75.59	
Mean =	23.2		50.34	56.53	60.60	65.98	72.05	77.74	

TABLE VIII—*Concluded*

($F_1 \times K$) Group 4 (i, II, III)

No.	Wt., *gms.*	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
2	22.8	*bl.h.*	53.46	60.36	61.58	62.88	75.50	79.91	82.00
14	26.0	*alb.*	53.93	55.82	61.94	65.95	75.06	76.00	79.44
16	28.5	*bl.h.*	54.12	57.75	—	64.93	69.84	77.39	78.33
17	26.5	*alb.*	52.87	55.03	59.38	63.82	73.76	78.94	79.10
20	19.5	*bl.h.*	53.28	59.53	61.21	64.68	76.05	—	82.25
Mean =	24.8		53.53	57.69	61.03	64.65	74.04	78.06	80.22

these in accordance with the indications of the presence of I, i; II, ii, or the other combinations. This has in fact been done. The alternative method is to consider the *cos* θ — *sin* α plots. The results of these two methods are completely concordant. Limitations of space lead us to present only the result of the latter proceedure in detail,but the material for the former treatment is given in Table VIII.

By very fortunate chance the races initially chosen for these experiments, *A* and *K*, turn out to be rather "simple", in the sense that our three "groups of sense organs" are each large, in *K*, small in *A*. Were it not for this fact the analysis would be much more difficult. As one result of the actual condition, the cos θ — sin α plots for these races (Fig. 1) are straight. But in a combination corresponding to I, ii, or to i, II, the graph with these coördinates should show a sharp break. With I, ii, the low - α end should be steep, as in the *K* race, the mid region less steep, as in *A* (Fig. 1). None-the-less, recognizing possibilities of errors in the determinations of θ with any one individual taken singly, it is possible to draw a fairly satisfactory line through the points for each one, plotted as cos θ *vs.* sin α. In the case of the *K* race, the slope of this line, in arbitrary units [4.0 Δ cos θ/(Δ sin α = 0.4)] is 12.6 units; for the *A* race, 7.8 units; for F_1 ($K \times A$) it is 8.4 units. We expect in ($F_1 \times K$) a group of individuals showing a cos θ — sin α slope near to 12 units, another with slope near 7.8; and, if the previous assumptions are just, two other groups with slopes in between these values. The relatively greater uncertainty (experimentally, not statistically,) of θ at $\alpha = 70°$ makes the finer separation

doubtful, since this θ is likely to be low. At least, we can say that one group should have the slope $\Delta \cos \theta/\Delta$ in α (chiefly weighed by the θ's at $\alpha < 70°$) near to that for *K;* another, more numerous, near to that for *A;* another, about as numerous as the first, with the slope intermediate. In fact the grouping by this method is rather rough,

TABLE IX

($F_1 \times K$)

Association of individuals in ($F_1 \times K$) as determined chiefly by the slope $\Delta \cos \theta/\Delta \sin \alpha$; as explained in the text, this is a rough measure only, and has to be corrected according to other criteria.

No.	$\frac{4\,0\,\Delta \cos \theta}{\Delta \sin \alpha = 0.4}$	No.	$\frac{4\,0\,\Delta \cos \theta}{\Delta \sin \alpha = 0.4}$
Group 1 (I, II, III)		Group 2 (i, ii, III)	
4	9 5	1	6.8
5	10 1	9	7 7
8	9 8	12	7.8
11	11 1	13	7 6
19	9.9	22	8.2
		21	7.1
Mean =	10 1	10	7.6
		Mean =	7.6
Group 3 (I, ii, III)		Group 4 (i, II, III)	
3	8 8	2	8 1
6	9 2	17	8.1
7	9 0	20	8 0
15	8.1	14	8.2
18	9 0	16	6.7
Mean =	8 8	Mean =	7.8

although quite clear, as Table IX shows; it has been checked by the more detailed consideration of the $\cos \theta - \sin \alpha$ plots for each individual, where it is clear that the slopes for the parts of the graphs compel the recognition of independent assortment of I, *i* and II, *ii* "groups of sense organs." This mode of associating the individuals is exhibited in Table VIII. The mean θ's for each group are plotted in

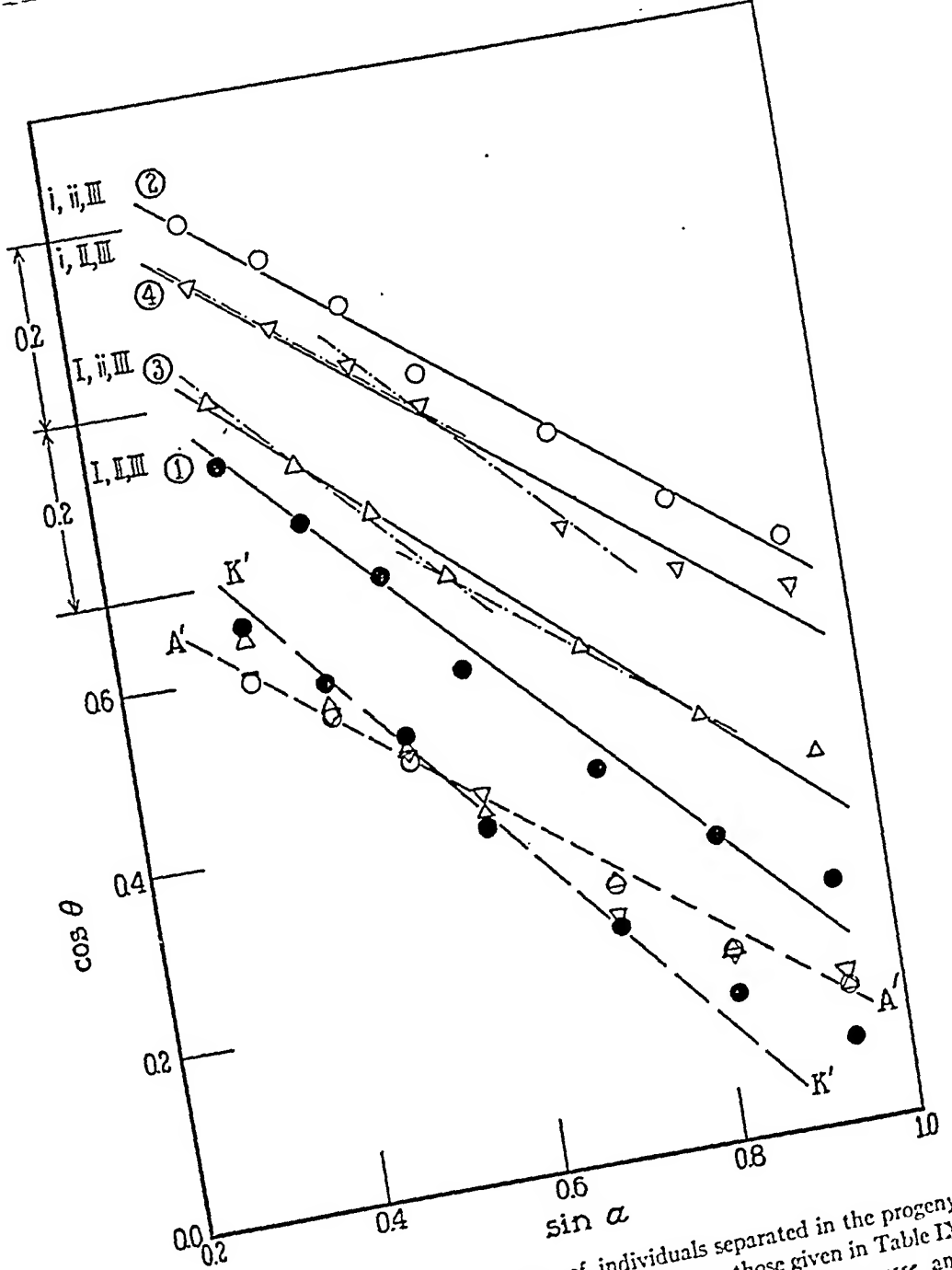


FIG. 19. Cos θ *vs.* sin α for four groups of individuals separated in the progeny obtained from ($F_1 \times K$). The slopes of the fitted lines are those given in Table IX (see text). In the lower part of the Figure the points are plotted *en masse*, and (with the shifts, A', K', discussed in the text) the lines for the grandparental families A and K are seen to include between them the scatter of all the data from the back-cross segregates. In the upper portion of the Figure the four groups of back-cross individuals are treated separately. The probable errors are less than the diameters of the symbols. In fitting the unbroken lines, relatively less weight has been given θ at $\alpha = 70°$; this point is the most likely to be untrustworthy.

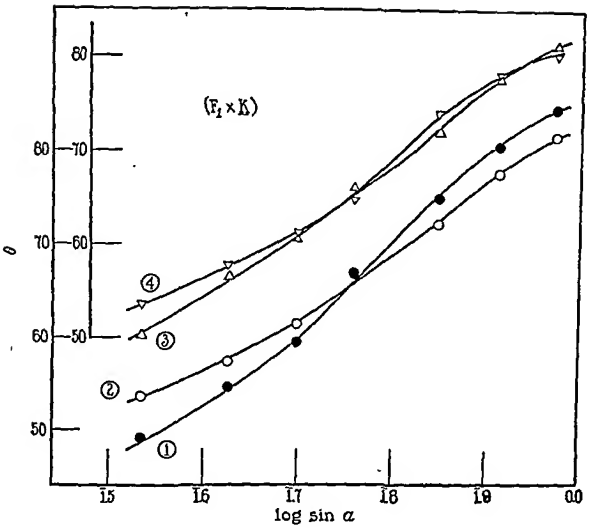


FIG. 20. θ vs. log sin α for the four groups of individuals segregated in the back-cross progeny ($F_1 \times K$). Group 1 should be like the grand-parental K; Group 3 should have its low-α end like that for 1, its mid-portion like that of Group 2. Group 2 should resemble closely, except at $\alpha = 55° - 70°$, the grand-parental A, or more closely the Group (i, ii, III) of segregates in the back-cross ($F_1 \times A$), with which its curve is indeed identical. Group 4 should show a lower portion of its curve like that in the curve for Group 2, its later portion like that in the curve for Group 1. In other words, as regards the slopes of these curves, considering each curve divided into two parts on either side of $\alpha = 35°$, we should find all possible combinations of low and high slopes—high slope below 35° combined with either high or low slope above 35° (up to $\alpha = 55°$), and reciprocally. It is clear that these combinations are indeed found.

Fig. 19, where the slopes given to the lines are those obtained in Table IX. When the separate series are studied it is evident that the "broken" character of the plots is sufficiently clear in the case of three of

TABLE X

The variability function (*cf.* Table II) for certain individuals from the back-cross ($F_1 \times K$).

The value for Nos. 8, 11 might be expected to be like that for *K:*

	θ, mean, degrees						
α =	20°	25°	30°	35°	45°	55°	70°
Nos. 8, 11	49.70	53.15	59.18	69.72	72.90	81.69	84.17
$100 \frac{\text{P.E.}_\theta}{\theta}$	2.7	2.0	2.21	1.42	1.14	0.80	0.63

The slope $\Delta \frac{\text{P. E.}\theta}{\theta} \times 100 / (\Delta \log \sin \alpha)$ is in this case, using units previously employed, = 2.00; N = 3.88; the product = 7.76, obviously of the order of magnitude obtained with *K* (*cf.* Table II).

Individuals 9, 10, 12, 13, placed in our second group, *i.e.* resembling the F_1 population, were chosen because 20 observations were obtained with each of these; the variability function is obtained from P. E. $/\theta \times 100$ for the summed records:

α =	20°	25°	30°	35°	45°	55°	70°
$100\ \text{P.E.}_\theta/\theta$ =	1 67	1 46	1 56	0 60	0 72	1 08	0.75

is $0.9 \times 0.71 = 0.64$

This compares sufficiently well with the value 1.92 (Table II) gotten with F_1, as to order of magnitude.

The cogency of this reasoning is further supported by analysis of the variability of the group of ($F_1 \times A$) individuals (Table II), determined to be like race *A*. For these, the value obtained is 2.5, which as to order of size agrees well with the value for *A* as gotten in Table II.

the four sets. The remaining one is like the original *K* line, save that the θ's are uniformly higher (cos θ's less). This corresponds to a uniform lowering of the *K* graph, with only very slight change of slope. The same shift is seen in the graph for the *A* line which "ought" to

describe the course of the observations with the second set (i, ii)below $\alpha = 50°$. It will be noticed that the graphs for the four sets cross at $\alpha = 36°\pm$, as predicted in an earlier Section (p. 78), and that the mean θ's are included within the lines corresponding (with the parallel shifts just mentioned) to the *K* and *A* grandparental races. The shifts correspond, it seems to us, to the entrance of other (genetic?) factors not segregating with I, i, etc., but capable of affecting θ; they cannot be associated simply with weight of individual; the effect upon the distribution curves for $\Delta\theta/\Delta \log \sin \alpha$ is to cause them to broaden out, with lowering of the ordinates (*cf.* Fig. 20, 21), as in the case of our *B* race.

An important check upon the associations given in Table VIII has been obtained by various trials at random and obviously "false" groupings. It was pointed out much earlier (p. 78) that in the total (F_1 x *K*) population P. E. θ/θ passes through a minimum at $\alpha = 36°\pm$, plainly indicating heterogeneity. For each group in Table VIII the variability of θ declines as a straight-line function of log sin α. With sundry false groupings this is at best only feebly true, and usually quite untrue. A striking point about the groupings here given is that for the segregates expected to be like the *K* grandparents the variability is so likewise; while for those expected to be like F_1 (*K* x *A*) this is also the fact. The *average* θ's given in Table VIII include for some individuals $20\pm$ observations each at each α, for others $40\pm$; the latter were obtained in the course of tests designed to see if facilitation ("learning") influenced the measurements of θ — as we have already discussed (p. 60). To simplify the presentation we choose two individuals of the group I, II. III and four from i, ii, III, and in Table X give the variability function for the two lots. If the *variability* is a function of specific sense organ groups, the "variability number" for the first lot should be like that for *K*; of the second lot, like that for F_1 (*K* x *A*). Table X shows that to a remarkable degree this is indeed the fact, although we do not seek to stress the conclusion unduly until further data are available.

If there is free association of I, i, II, ii, and of the color-factors involved in the cross, we expect equal numbers of I and of i, of II and of ii, and of *black hooded* and of *albino* individuals, with no indication of connection between color and geotropic

there are in Table VIII respectively ten and twelve cases; of II, ii, respectively ten and twelve; of the former (*cf.* Table VIII) nine are *bl.h.*, thirteen *alb.;* of the latter, the ratio is the same. Table XI shows no connection between *color* and geotropic response. As stated in discussing F_1 ($A \times K$), this fact we regard as direct evidence that no untoward influence dictated our selection of litters for examination, since the inheritance of the color genes is well known, and the appearance of the patterns in our individuals shows chance arrangement.

When θ is plotted against log sin α, we expect for these groups of individuals curves of which the differentials should show, when plotted

TABLE XI

Distribution of the "sense organ groups" I, i; II, ii in relation to color factors, in the progeny ($F_1 \times K$).

	bl.h.	*alb.*	
I	3	7	*10*
i	6	6	*12*
	9	*13*	
	bl.h.	*alb.*	
II	5	5	*10*
ii	4	8	*12*
	9	*13*	

against sin α, the 3 "groups of sense organs" previously recognized. Of the θ-curves we expect one to be like that for F_1, one to be like that for K, the other 2 different in certain predictable ways. At the same time, the shift of the cos θ graphs we have earlier remarked as "flattening" the differential curves. This corresponds to a curious "rotation" of the θ — log sin α curve, about a mid-point, as seen also in the comparison of lines A and B, and may be related to the participation of other ("genetic"?) influences, but we are in no position to discuss these concretely. However, there is no difficulty in recognizing the four kinds of individuals we in this case seek. It is instructive to notice

that several of these sorts of individuals give θ — log sin α graphs much more nearly straight, as a first approximation, than those originally gotten for races *K*, *A*, *B*, etc. This again reminds one of the dangers of "Weber's Law," and leads us to remark that this particular straight-line is not quite the same thing as that usually appealed to in such relations. In the present cases the approximate rectilinearity holds over the whole workable range of α, whereas it is well known that the relation usually seen between response and log stimulus, and for photic excitation clearly analyzed by Hecht (1924, *b*), holds only over the midrange.

Curves of θ *vs.* log sin α for the four sorts of individuals recognized in (F_1 x *K*) are plotted in Fig. 20. The differences among these have already been briefly characterized. It may be noted that the probable errors of the mean θ's are less than the diameters of respective symbols, but are a little larger than might be expected on the basis of the numbers of readings, owing to the fact that the θ curves for two individuals may be parallel, but $\Delta\theta/\Delta\alpha$ the same; the use of litter-mates in the *K* and *A* series apparently avoided this effort.

A general scheme for the inheritance of these phenomena can accordingly be written:

Receptor groups	1	2	3
Race A.	i	ii	iii
Race K	I	II	III
F_1, A × K	*i(I)*	*ii(II)*	*III(iii)*,

with the realization that in backcrosses with *A* and with *K* the expected phenotypic classes, recognizable by several different tests, in each case appear to be found, and in proportions of individuals which clearly indicate the possibility of independent alternative inheritance of the factors recognizably concerned.

Genetically, the amount of geotropic orientation in these animals must be regarded as a "multiple factor" effect. It is possible to foresee that in certain other instances it may be feasible to disentangle the relations of several cooperating genes by the similar application of methods which seek to modify quantitatively the expression of composite resultants according to the influence of some controlling condition of the respective contributing factors.

VI

With regard to the possible significance of these differences, interpretation must proceed slowly. They cannot be accounted for by mere differences among the weights of the several individuals. Since, as we have seen, the values of θ at threshold α are apparently distributed in a way corresponding, at least roughly, to the finer differences brought out, it might be conceived that *re-distributions* of the weight, in terms of differential growth rates of the regions of the body, might influence in a differential way the excitation of a fundamentally uniform series of receptors,—essentially the same, that is, in all the cross-bred individuals. But a little reflection makes it appear that this assumption would require rather elaborate hypotheses if it should attempt to explain in detail the effects as found, and it could not interfere with the conclusion we are impelled to draw.

If we accept the initial assumptions of these experiments, chiefly that according to which θ is determined by the achievement of essentially equivalent excitation of tension receptors on the two sides of the body, θ in degrees being therefore a proportional statement of the total amount of excitation, we must recognize at least the following elements in the case: (1) the magnitudes of the exciting pull ($k \sin \alpha$); (2) the variation of excitation thresholds among the population of receptors; (3) the frequency with which their excitation occurs; and, presumably, (4) central nervous thresholds. The relative simplicity of the results obtained in endeavoring to analyze the observations encourages us to believe that the method of treatment deals successfully with (1) and (2–3). It is implicit in the treatment that by a "receptor" effect we include the whole chain of events from sense organ through to central organ and beyond, and include also the frequency of excitation,—because it is in these terms that our "groups of receptors" are recognized. But it should be noted that the speed with the A line is, at given α, a little *less* than with K; qualitatively it increases in the same way. We have ignored possible differences in speeds of creeping, because no great absolute differences were perceptible between the several lines used; and there is at present no means of dealing with the significance of the speed as related to length of limbs and the like. The matter of "central thresholds" ((4) above,) we cannot deal with directly. In view of the correlation between

threshold θ's and the slopes of the θ — log sin α lines, it might be supposed that the essential genetic differences between lines, K, A, B, and the $K \times A$ hybrids relate merely to the ease with which particular assemblages of tension-receptors become effective as loci of stimulation, whether through differences in central nervous conditions, or through differences in the relative excitabilities arising through diverse arrangements of the body mass relative to the dimensions or attitudes of the appendages during creeping, or to the frequencies of stepping. Such differences might well be responsible for the fact that at the slope where θ begins to be exhibited as a definite function of the slope, different "numbers of receptors" are already involved in the several cases. The question remains, whether these additional receptors are drawn from the whole available array, or selectively from one or more groups of them. But in neither case would the effect in any way minimize the significance of the recognition in the present cases of *three* such groups. As is shown in the final section of this paper, a selective effect on *one* of these three groups can be demonstrated when an additional mass is appropriately carried by the creeping rats. A choice among the several alternatives suggested (and others are possible) would affect merely the *interpretation* of the genetic results. In other words, we cannot say that the genetic differences utilized and revealed in the present experiments definitely depend upon the occurrence of three distinct groups of sense organs, the number of organs in each group differing in the several pure lines used and being subject to the rules of simple alternative inheritance, *because the conditions of their effective excitation may be the things subject to inheritance.* Whether these conditions are of a central nervous sort, or depend merely upon the relative rates of development of the numbers of tension receptors in our three groups, in the several races used, cannot be stated. The contrast between lines B and A might be understood in either way. These considerations do not affect the fact (1) that the quantitative differences found between lines A and K are shown by their genetic analysis to be *real* differences, and (2) that genetic differences of the sort we have used can be recognized and characterized only by quantitative procedures which seek to define the genetic phenomenon as a function of an experimentally controllable variable.

VII

It has been clear to us that the existence of the three "groups of sense organs" apparently concerned in the adjustment of the geotropic orientation of our young rats might be made more certain if demonstrable by some independent test. At the same time, it might be possible to predict upon this basis, at least in a *general* way, the outcome to be expected as result of certain modes of experimental manipulation. If, for example, our three categories of sense organs inhabit diverse organic loci,—muscles, tendons, skin,—it might be possible by increasing the loads to be carried by the young rats to modify in a

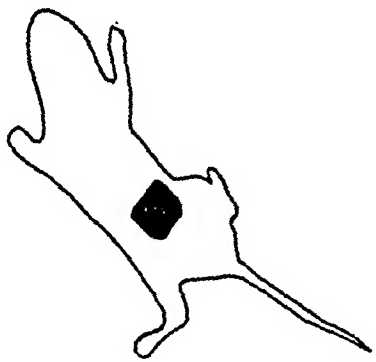


FIG. 21. Outline of a rat creeping on an inclined surface, with flat brass weight attached to the skin of the back. Outline from motion-picture record.

predictable way the curve connecting θ with α. We have earlier demonstrated (Crozier and Pincus, 1926–27,*a*; Pincus, 1926–27) that when small masses are attached to the base of the tail of these animals, at constant α there is evident an increase of θ about proportional to log m, where m is the added mass, and that the *speed* of linear progression is similarly influenced. The information now required, however, depends upon measurements of θ as a function of α, with m constant. If the added weight is so located as to influence primarily *one* of our "three groups of sense organs," bringing it into play at lower values of α than in the absence of the weight, then we expect the curve connecting θ with log sin α to be distorted at one end or the other. In that event, curves of the type of those already

shown in Figs. 10, 11, should undergo a change signifying a shift of *apparent* thresholds of the sense organs of one or more groups.

An experiment of this sort may be described here. It was made with individuals of race *K*, under standard conditions, the individuals first being tested for orientation at several values of α and then employed after a flat brass weight (2 gms.) had been attached to the center of the back with a thin layer of chicle. The total attached mass was

TABLE XII

Angles of upward orientation (θ) of young rats of line *K*, carrying a mass of 2.15 gm. as shown in Fig. 21, at various inclinations of the surface (α). Two series of tests (i, ii), with four individuals ($n = 40$) in each. The way in which θ is increased, at lower values of α, is made clear by Fig. 22. The manner in which the $\Delta \cos \theta / \Delta \sin \alpha$ relationship is disturbed is shown by Fig. 24.

α degrees	θ degrees	
	(i)	(ii)
15	51.56 ±1.38	49.80 ±1.20
20	59 65 ±1 32	57.76 ±1.09
25	66 80 ±1 07	63.71 ±0.92
30	70 10 ±0.94	
35		69.20 ±0.87
40	70 83 ±1 01	
50	78.41 ±0 80	
55		78.91 ±0.71
65	85 15 ±0 55	
70		86.12 ±0.43

2.15 gm. It is not particularly easy to attach weights to these animals, on account of the looseness of the skin, and the method now used was adopted only after trial of a number of others involving the use of tiny harness straps (of rubber dam), clamps, and the like. The diagram in Fig. 21 illustrates the relation of the weight to the animal's axis.

The experiment comprised two parts. In the first, measurements of orientation were obtained at various inclinations in about 10 runs with each of four individuals. Care was taken to exclude fatigue

effects. The second part of the test was a repetition of the foregoing with a new litter of rats, some days later.

The results from these two experiments are collected in Table XII. The plot in Fig. 22 makes it evident at once that at low values of α, θ is increased, in a regular manner, by the presence of the added mass.

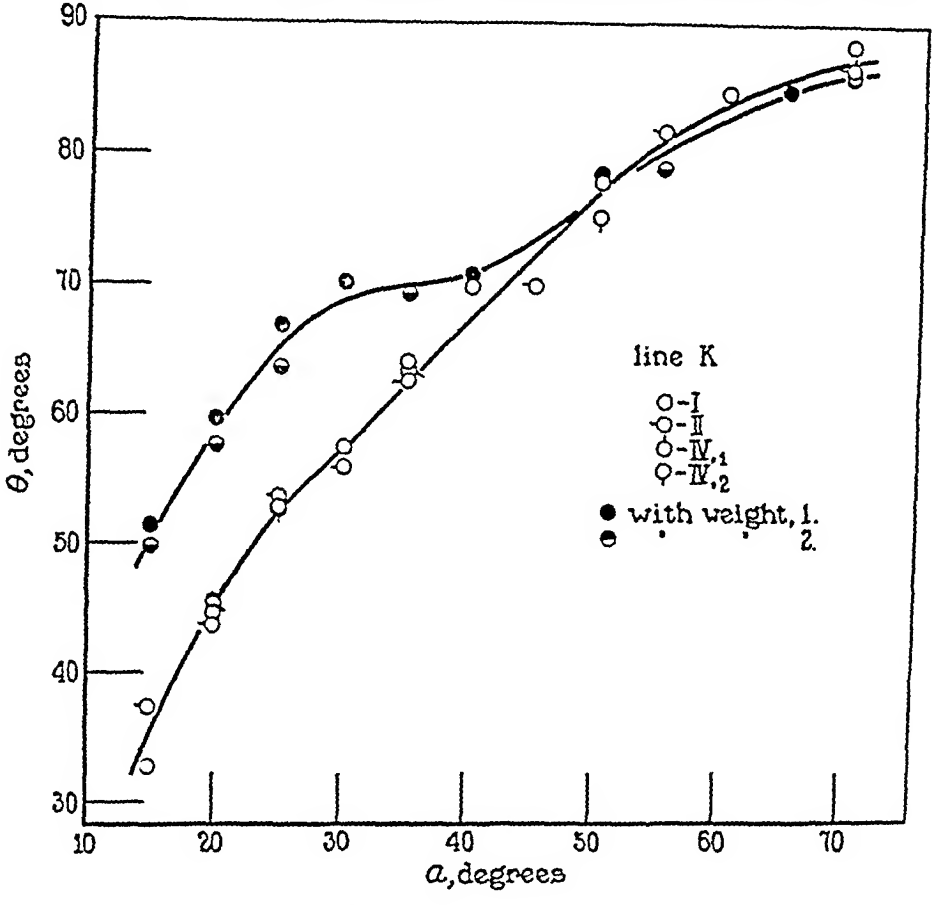


FIG. 22. Orientation-angles (θ), at different slopes of surface (α), for individuals of line *K* with added load (2.15 gms.); lower curve, without weights.

Moreover, the flatness of the curve at intermediate magnitudes of log sin α clearly suggests the kind of unequal distortion of the θ curve which we have seen reason to expect. The differential curves corresponding to the θ-graphs in Fig. 23, obtained as previously described in connection with Figs. 10, 11, are given in Fig. 25. If the amount and the adjustment of the weight in these tests has been such as to

influence *chiefly* the sense organs of our group II, and to a lesser extent those of group I, bringing the affected ones into play at low angles of inclination, below or as low as at the working threshold inclination, then the area under the differential curve *B* in Fig. 25 should be less than, and proportionately related to, the area under curve *A* in Fig. 25. It is evident that this is in fact the case. The mean θ, without

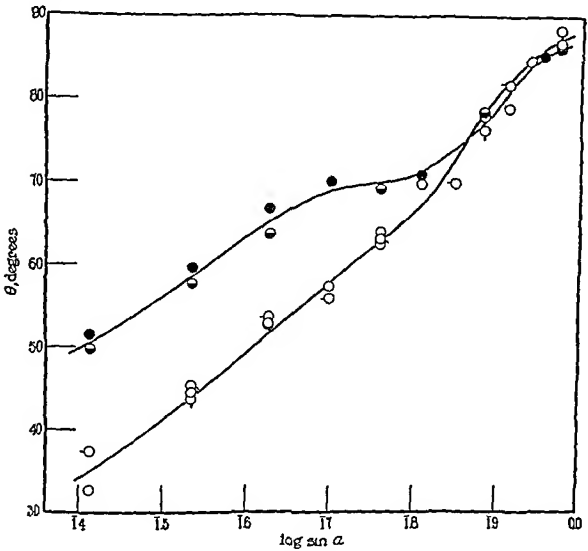


FIG. 23. θ vs. *log sin* α, with and without added load (2.15 gms.); race *K*.

weight added, at 15°, is (from the curve) 34°; with a load of 2.15 gms., $\theta = 50°$. Continuing the assumption that $\theta \equiv$ the total number of sense organs capable of controlling the geotropic orientation, and assuming that *all* these are activated when $\theta = 90°$, then the number remaining to be activated by increasing α above 15° is, without the weight, proportional to 90−34, or 56; with the load, to 90−50, or

40; in the latter case the number of "available sense organs" is then reduced in the proportion of 40/56, or of 2.0/2.8. Actually, the areas under the two curves in Fig. 25 are (in arbitrary planimeter

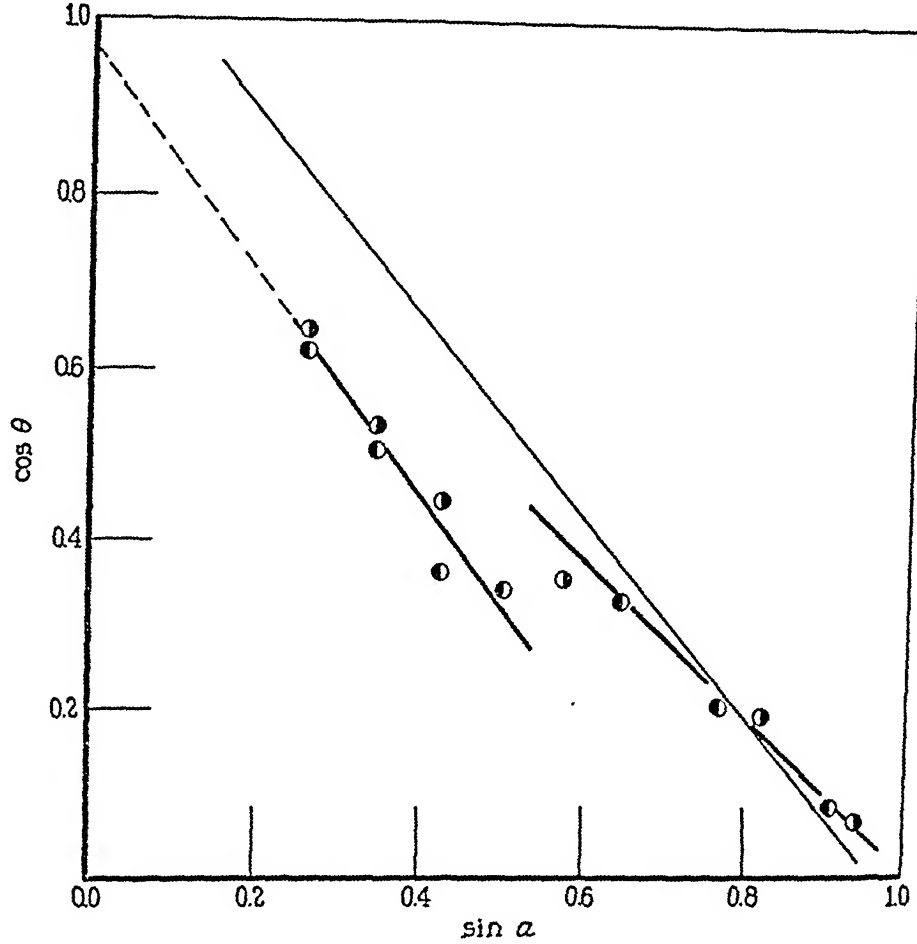


FIG. 24. *Cos θ* vs. *sin α* for line *K* individuals with added load. The original *K* graph is added as a thin line. Note that the projection of the initial segment of the broken graph goes practically through the origin ($\alpha = 0$, $\cos\theta = 1$); the downward displacement is a function of the added mass.

units) as 24.90 to 16.77, or in the ratio of 2.0/2.98. *If we assume that* all the activation effectively possible is obtained at $\theta = 83°$, then the ratio (83 − 34) / (83 − 50) is as 2.0/2.97. This *is* effectively the maximum obtainable θ. The agreement is so close as to preclude accident,

we believe; if this be correct, there results a striking justification of the conception that the orientation angle is determined in such a way as to reflect, directly, the total number of receptors activated per unit of time during oriented progression. It will be noted that on the *cos* θ plot (Fig. 24) the extension of the line appropriate to the observa-

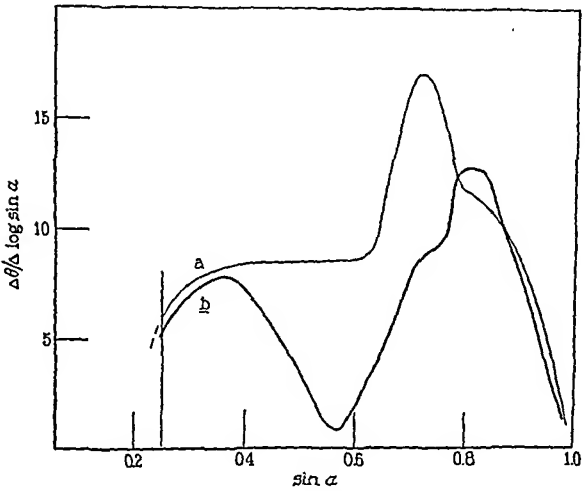


FIG. 25. $\Delta\theta / \Delta \log \sin \alpha$, vs. sin α, for *K*-line individuals carrying weights (*a*); for *K*-rats without weights, thin line.

tions at lower values of θ passes practically through the $\theta = 0$ origin (*cos* $\theta = 1$). This would be interpreted to mean that the presence of 2.15 gms. added mass stimulates none of the receptors in question while the animal is moving in a horizontal plane. The modifications of the curves which might be expected with larger masses, or by employing rats of other races, cannot be entirely foreseen.

The variability of θ, as P.E. $_\theta/\theta \times 100$, is much reduced, propor-

tionately, at equivalent α, for individuals carrying an added load (2.15 gms.), as shown in Fig. 26 A. By comparison with a series (open cir-

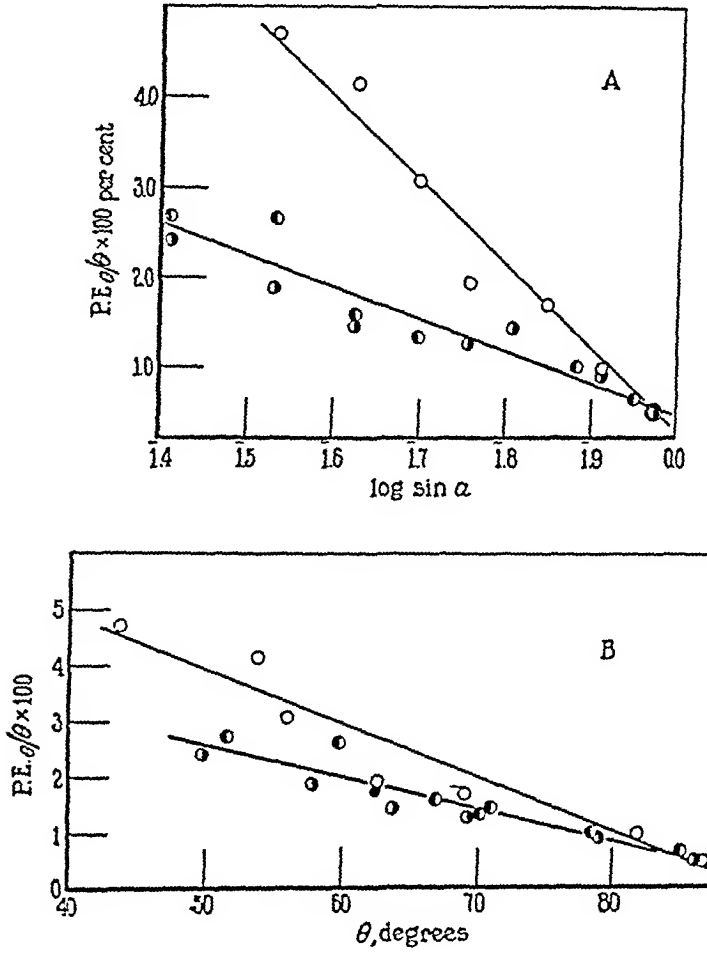


FIG. 26. *A*. The percentage variability of θ, as P. Γ $/\theta \times 100$, is much reduced, proportionately, at equivalent α, for individuals carrying an additional load (2.15 gms.), by comparison with series *K* II (open circlets) where the same numbers of individuals and of observations are concerned.

B. The variability of θ plotted against θ, for the two sets of observations. See text.

clets) in which the same number of individuals (4) was concerned, with the same number of readings on each, the slope Δ (P.E./θ)/Δ

log sin α is in the two cases in the ratio of about 1:2.57. If the able errors, as percentages, are however plotted against θ for series, as in Fig. 26 B, the relationship is again sufficiently rectili and the comparison is fairer. The slopes of the fitted lines a 1:1.66. If we assume the variability, with the load, to be reduc proportion to the effectiveness of the total load as a gravitatio activated stimulating agency, we might expect the variability ceptible to reduction by increasing α to be diminished in proporti the θ angles at threshold α (15°); this is as 51:34, or as 1:1.5. agreement with the observed 1:1.66 is suggestive, and suppl further confirmation of the analysis.

There is obtained in this way a distinctly encouraging proof the elements underlying the assumption of definite geotropic ori tion under conditions of steady progression, which have been d ciated by purely genetic methods, can by an independent metho shown to occur as distinct entities. We do not now hazard an opi as to the possible structure character or the locations of the di "groups of sense organs." It is possible that weights attached t rats at other parts of the body would produce qualitatively diff effects; indeed, this would be our expectation. But we submit the general results of this analysis, including the outcome of the a cation of the independent tests which have been described, consti a reasonably self-consistent picture; and that alternative interp tions of the facts which we have adduced must be prepared to acc for these relationships in a definite way.

VIII

SUMMARY

1. Equations describing the geotropic orientation of young as a function of the inclination of the surface on which creeping t place, under standardized conditions, are found to be of similar f but with different values of the contained constants, when sev different, genetically stabilized lines or races are compared. values of these constants are characteristic for the several races.

2. The biological "reality" of the differences between young of two races, as given mathematical form in terms of these parame

and coefficients, can be submitted to radical test by investigating their behavior in inheritance. A simple result favorable to the inquiry would be decisive; a complex, non-clear result would not however be definitely unfavorable to the view that "real" differences in behavior are in question. The actual result is of a kind demonstrating (a) the efficiency of the original formulations, and (b), at the same time, the definite inheritance of certain quantitative aspects of geotropic behavior.

3. On the assumption that orientation on a sloping surface is achieved when, within a threshold difference, the tension-excitations on the two sides of the body (legs) are the same, the angle of oriented progression (θ) can be taken as a direct measure of the total excitation. This is consistent with the equation, accurately obeyed by our initial races, $\Delta \cos \theta / \Delta \sin \alpha = -$ *const.*, where α is the slope of the surface.

4. The total excitation of tension-receptors must be regarded as involving, over a gross interval of time, (1) the total array of receptors with thresholds below a certain value, a function of the stretching force, and (2) the frequency of change of tension. The latter, largely determined (it is assumed) by the frequency of stepping, should be proportional to the speed of progression. This speed is directly proportional to *log sin* α. Hence $\Delta\theta/\Delta \log \sin \alpha$, plotted against *sin* α, should give a picture of the distribution of effective thresholds among the available tension-receptors in terms of the exciting component of gravity. For the races investigated this distribution can be resolved in each case into three groups.

5. A "variability number" is employed which permits the demonstration that the variability of θ as measured is (1) definitely controlled by α, and is (2) a characteristic number for each of the pure races used.

6. By attaching a weight to rats of one race it is found that $\Delta\theta/\Delta\alpha$ is modified in a manner concordant with the assumption that the three "groups of sense organs" are in fact discrete.

7. In race *K* these three groups (I, II, III) are large, in race *A*, small (i, ii, iii). F_1 rats of the cross between these two races show i, ii, III.

8. F_1 individuals back-crossed to *A* give in the progeny two sorts of individuals, in equal numbers: i, ii, III and i, ii, iii.

9. F_1 individuals back-crossed to K are expected to give in the progeny four types of individuals, I, II; i, II; I, ii; i, ii. In the numbers available these classes are reasonably clear, and occur with equal frequency.

10. It is pointed out that these considerations imply a mode of definition of a gene somewhat different from that commonly employed by tacit assumption; namely, a definition of the effect in inheritance as a function of some controlling, independent variable.

CITATIONS

Adrian, E. D., 1928. The Basis of Sensation; The Action of the Sense Organs, London, New York.

Adrian, E. D., and Zottermann, Y., 1926, *J. Physiol.*, lxi, 151.

Allen, F., and O'Donoghue, C. H., 1927, *Quart. J. Exp. Physiol.*, xviii, 199.

Cooper, Sybil, and R. S. Creed, 1926–27, *J. Physiol.*, 62, 272.

Crozier, W. J., 1924–25, *J. Gen. Physiol.*, vii, 189. 1925–26, *J. Gen. Physiol.*, ix, 531. 1928, *J. Gen. Physiol.*, i, 213.

Crozier, W. J., and Navez, A. E., 1930, *J. Gen. Psychol.* (In course of publication.)

Crozier, W. J., and Oxnard, T. T., 1927–28, *J. Gen. Physiol.*, xi, 141.

Crozier, W. J., and Pincus, G., 1926, *Proc. Nat. Acad. Sci.*, xii, 612. 1926–27*a*, *J. Gen. Physiol.*, x, 257. 1926–27*b*, *J. Gen. Physiol.*, x, 407. 1926–27*c*, *J. Gen. Physiol.*, x, 419. 1926–27*d*, *J. Gen. Physiol.*, x, 519. 1927–28, *J. Gen. Physiol.*, xi, 789.

Crozier, W. J., and Stier, T. J. B., 1924–25*a*, *J. Gen. Physiol.*, vii, 429. 1924–25*b*, *J. Gen. Physiol.*, vii, 705. 1925–26, *J. Gen. Physiol.*, ix, 547. 1926–27, *J. Gen. Physiol.*, x, 501. 1927–28, *J. Gen. Physiol.*, xi, 803. 1928–29*a* (In course of publication). 1928–29*b*. (In course of publication.)

Crozier, W. J., and Wolf, E., 1928–29, *J. Gen. Physiol.*, xii, 83.

Hecht, S., 1924*a*, *J. Gen. Physiol.*, vi, 355. 1924*b*, *J. Gen. Physiol.*, vi, 235. 1927–28, *J. Gen. Physiol.*, xi, 255.

Hoagland, H., 1929, *J. Gen. Psychol.* (In course of publication.)

Hovey, H. B., 1928, *Physiol. Zoöl.*, i, 550.

Hunter, W. S., 1927, *Ped. Sem.*, xxxiv, 299.

Keeler, C. E., 1927–28, *J. Gen. Physiol.*, xi, 361.

Kropp, B., 1929, *J. Gen. Psychol.* (In course of publication.)

Kropp, B., and Crozier, W. J., 1928–29, *J. Gen. Physiol.*, xii, 111.

Loeb, J., and Ewald, W. F., 1911, *Biochem. Zeits.*, lviii, 177.

Morgan, T. H., Sturtevant, A. H., Muller, H. J., and Bridges, C. B., 1922, The Mechanism of Mendelian Heredity (Rev. edn.) New York.

Piéron, H., 1928*a*, *Ann. Physiol. et Physicochem. Biol.*, 4, 44. 1928*b*, *J. Genetic Psychol.*, xxxv, i.

Pincus, G., 1926–27, *J. Gen. Physiol.*, x, 525.

Szymanski, J. S., 1920, *Zeits. f. allgem. Physiol.*, xviii, 105. 1922, *Zeits. f. angew. Psychol.*, xx, 192.

Wickham, H., 1928, The Misbehaviorists. New York.

Wolf, E., 1926–27, *J. Gen. Physiol.*, x, 757.

Wolf, E., and Crozier, W. J., 1927–28, *J. Gen. Physiol.*, xi, 7.

PROTEIN COAGULATION AND ITS REVERSAL

THE PREPARATION OF COMPLETELY COAGULATED HEMOGLOBIN

BY M. L. ANSON AND A. E. MIRSKY

*(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.)**

(Accepted for publication, June 27, 1929)

Irreversibility has always been considered one of the most striking characteristics of the coagulation of proteins. Four years ago, our experiments with hemoglobin led us to suppose that coagulation is reversible (2).† More complete evidence that the coagulation of hemoglobin can actually be reversed is provided by the present experiments. This first paper describes the preparation of completely coagulated hemoglobin and shows that the denaturation and coagulation of hemoglobin are the same as the denaturation and coagulation of other proteins. The second paper of this series describes the preparation from completely coagulated hemoglobin of soluble crystalline hemoglobin which by all the tests tried has been indistinguishable from the original native hemoglobin. Other papers are concerned with (1) the properties of proteins coagulated at interfaces, (2) the preparation of so-called native globin and the reversibility of the coagulation of globin, and (3) some properties of the tissue proteins and their significance in such phenomena as muscular contraction.

Description of Denaturation. As has been shown by Hardy (7),

* Many of the experiments described in this and the following paper were done in the Biophysics Laboratory of The Cancer Commission of Harvard University. A brief account of these experiments was presented to the Society of Biological Chemists in 1927 (3). Our first preparation of crystalline hemoglobin from coagulated hemoglobin was described in our doctoral dissertations, University of Cambridge, 1926.

† Wu and Lin (15) have confirmed these early experiments but they disagree with our interpretations of them. Spiegel-Adolf (13) has recently presented evidence that the denaturation of serum albumin is reversible.

Chick and Martin (4, 5), and Sørensen (12), coagulation proceeds in two distinct steps. The first step, known as denaturation, is a change in the native protein brought about by heat, acid, alcohol and other agents,* which makes the previously soluble protein insoluble around its isoelectric point. The second step in coagulation is the precipitation of the insoluble denatured protein. Denatured protein although insoluble around its isoelectric point is soluble in acid or alkali. If, therefore, a protein is denatured in a nearly isoelectric solution, a visible precipitate results. But if a protein is denatured in acid or alkaline solution, no visible change results, until the solution is made isoelectric—when the protein is precipitated. The second step in coagulation, the flocculation of the insoluble protein, is, as has long been known, reversible, since the flocculated protein may readily be redissolved. The first step in coagulation, denaturation, has hitherto not been reversed. When a solution of the coagulum in acid or alkali is brought to the isoelectric point of the protein, the protein is again precipitated; it is still denatured. Denaturation, therefore, is the important process in the investigation of the reversibility of coagulation.

Denaturation is not a general disintegration of the native protein. Did denaturation involve general disintegration reversibility would be impossible. The theory that denatured protein is an indefinite, early breakdown product of native protein is made plausible by the fact that heat, acids, alkalies and some of the other agents that bring about denaturation also cause a general disintegration. Heat, for instance, splits off nitrogen. Sørensen (12), however, has decisively shown that this splitting off of nitrogen is incidental rather than essential to denaturation. Similarly, we have found that alkali denatures a protein thereby rendering it insoluble in water, but that this same alkali gradually alters the denatured protein so that it becomes more soluble in water. It will be shown in a future paper that a protein solution made more viscous by alkali denaturation is (after denaturation is completed) gradually made less viscous by the alkali. These secondary effects of the denaturing agents should be clearly distinguished from the denaturation itself.

* It has not yet been proven that the various forms of denaturation result in identical changes in the protein.

I. The Preparation of Completely Coagulated Hemoglobin

It must be shown that the denatured hemoglobin used in our experiments on the reversibility of coagulation is completely denatured, that it contains no significant impurity of unmodified native protein. The ultimate test for complete denaturation is by definition complete insolubility at the isoelectric point. The possibility must be considered, however, that completely precipitated protein may be only apparently completely insoluble, for it is conceivable that denatured protein when precipitated carries down soluble native protein. Experimentally, however, at least under such conditions as render it possible to have a known mixture of native and denatured proteins, the precipitation of the denatured protein does not result in the removal from solution of any native protein. Furthermore, in the case of heat denaturation the kinetics of the reaction make it improbable that soluble protein is being carried down by the coagulated protein. In addition to solubility observations, there are two independent tests for complete denaturation. First, the same yield of soluble apparently native hemoglobin is obtained from hemoglobin denatured by heat at two different temperatures. This could hardly be true did the soluble hemoglobin come from any hypothetical residue of undenatured protein. Secondly, the denaturation of hemoglobin in urea solutions is accompanied by viscosity changes. When the viscosity ceases to change, the denaturation is probably complete.

In the following preparations of denatured hemoglobin the protein has been denatured in four different ways, by heat, acid, heat together with acid, and urea. In all four cases the completeness of denaturation has been tested by solubility observations. Wherever possible, the independent tests have also been used.

It is important to note that 30 per cent of the coagulated hemoglobin is finally obtained in the form of soluble, crystalline, apparently native hemoglobin. The existence of a trace of native hemoglobin in our preparations of coagulated protein would be insufficient to account for such a result.

(*1*) *Acid.* The first experiment describes the test for complete insolubility. The evidence is then given that native protein is not carried down.

1.3 cc. of a 10.85 per cent solution of horse hemoglobin are warmed to 40°C. To it are added 2 cc. $\frac{N}{5}$ HCl likewise at 40°C. and the two solutions are thoroughly mixed and kept at 40°C. for 3 minutes. The hemoglobin solution is now chocolate brown instead of deep red. To this solution is added a mixture of 3 cc. $\frac{N}{5}$ NaOH and 2 cc. $\frac{M}{5}$ KH_2PO_4 warmed to 40°C. The pH is now about 6.8, close to the isoelectric point of hemoglobin and in the region of minimum solubility of denatured hemoglobin. The flask is well shaken so as to facilitate subsequent filtration. The filtrate is clear and practically colorless. The nitrogen content of the filtrate is somewhat less than 0.05 mg. per cc., an amount that is just measurable. This experiment is carried out at 40°C. to facilitate filtration. When done at room temperature the filtrate is cloudy; at 40°C. it is clear. 40°C. is far below the coagulation temperature of hemoglobin in water, which is between 70° and 80°C.

To see whether native hemoglobin is carried down by denatured protein, the denatured protein is precipitated from a known mixture of native and denatured hemoglobin and the native protein estimated in the filtrate. The known mixture is prepared by adding a known amount of native hemoglobin to a known amount of denatured protein dissolved in acid. The pH and temperature must be such that the native protein is not denatured during the time of the experiment. Under these conditions no evidence of carrying down of native protein has been found. What would happen under conditions under which rapid denaturation takes place is not open to direct experiment.

To 10 cc. of the 10.85 per cent hemoglobin solution are added 20 cc. $\frac{N}{5}$ HCl and the mixture allowed to stand for 3 minutes. 20 cc. $\frac{N}{5}$ NaOH are added. The suspension is centrifuged, the supernatant fluid rejected, and water added. It is shaken and centrifuged again. To the suspension of denatured hemoglobin 3 cc. $\frac{N}{5}$ HCl are added. This dissolves most of the protein. The suspension is centrifuged to remove the undissolved portion. The volume of solution is now about 10 cc. and contains about 4.2 per cent protein. 0.5 cc. of 13.8 per cent of carbon monoxide hemoglobin is mixed with 4 cc. $\frac{M}{5}$ KH_2PO_4 and warmed to 40°C. Carbon monoxide hemoglobin is used in this experiment because it is more stable in acid than is either methemoglobin or oxyhemoglobin. This HbCO solution is added to 2 cc. of the solution of denatured hemoglobin in HCl. Immediately

afterward 2.6 cc. $\frac{N}{5}$ NaOH warmed to 40°C. are added. The mixture is well shaken and then centrifuged. The supernatant fluid has the color of HbCO and its concentration can be estimated colorimetrically. Within the error of the experiment (about 3 per cent) all of the HbCO added is recovered, that is to say, no native protein is brought down by the coagulum.

It is conceivable that methemoglobin or free native globin might behave differently from HbCO, so experiments have also been done with these proteins.

A solution of horse methemoglobin is prepared by adding potassium ferricyanide to an oxyhemoglobin solution until the absorption bands of oxyhemoglobin can no longer be seen. This solution is dialyzed against distilled water to free it from ferricyanide. After dialysis the protein concentration is 12 per cent and the specific conductivity is 1.4×10^{-4} reciprocal ohms. The methemoglobin is diluted to 5 per cent with distilled water. To 5 cc. methemoglobin are added 1 cc. $\frac{N}{10}$ HCl and 0.5 cc. $\frac{M}{2}$ NaCl. The solution is heated in boiling water for 3½ minutes and then cooled at 0°C. It is added to 5 cc. $\frac{M}{5}$ phosphate buffer (at 0°C.) of pH 6.8. The mixture is filtered and to the hazy filtrate half its volume of saturated $(NH_4)_2SO_4$ is added. This mixture is filtered, and the filtrate is now clear. This nearly colorless filtrate contains less than 1 per cent of the protein present before coagulation. The rest of the protein has been apparently coagulated. To see whether any methemoglobin is carried down by the coagulum the experiment is repeated but just before adding the buffer 1 cc. of 5 per cent methemoglobin is added. The final filtrate is now colored and its color is compared with that of a standard containing 1 cc. of 5 per cent methemoglobin and 17.25 cc. of the nearly colorless filtrate from the original coagulum. These two solutions are indistinguishable colorimetrically, indicating that no native protein is carried down by the coagulum.

If, instead of precipitating the denatured protein at its isoelectric point, it is precipitated from acid solution by ammonium sulfate it can be shown that no native protein is carried down by the coagulum. All of these experiments can be done, using slightly different quantities, with oxyhemoglobin. They can also be done using so-called native globin and denatured globin or hemoglobin. The result is always the same.

(2) *Heat.* When the coagulum is obtained simply by heating the

native protein, the filtrate contains practically no nitrogen. The record of an experiment is as follows:

To 7 cc. of a 10.85 per cent hemoglobin solution are added 10 cc. of water. The solution (in a test tube) is plunged into boiling water for 3½ minutes and then cooled in cold water. To the coagulum 6 cc. of a pH 6.8 phosphate buffer are added and mixed thoroughly with the protein. The suspension is filtered and the nitrogen content of the filtrate estimated by a micro-Kjeldahl method. Each cc. contains 0.065 mg. of nitrogen. If this were due to hemoglobin it would represent a solution of only about 0.035 per cent. This is negligible compared with the amount of soluble protein obtained from the coagulum.

In heat coagulation there is additional evidence that the coagulum contains no native protein and that the soluble protein does not come from such an impurity in the coagulum. Heat denaturation follows the course of a unimolecular reaction at all temperatures at which it can be measured (5). In experiments on the kinetics of this reaction the concentration of the reactant, native hemoglobin, is measured by filtering off the coagulum at any given time and estimating the hemoglobin concentration in the filtrate. To harmonize these facts with the assumption that native protein is carried down one must further assume that every molecule of denatured protein carried down a given and constant number of molecules of native protein.

(*3*) *Heat in Acid Solution.* The completeness of denaturation in this case can be tested not only by the observation of complete insolubility but also by a comparison of the yields of soluble protein obtained from hemoglobin denatured at two different temperatures. The denaturation of hemoglobin has a high temperature coefficient. If hemoglobin heated for 3½ minutes at 80°C. still contains some undenatured protein, then hemoglobin heated at 100°C. should contain at least a hundred times less. Did the soluble, apparently native hemoglobin obtained from the heat denatured hemoglobin come from undenatured protein, then one would expect to get much less of this soluble protein from the hemoglobin heated at 100°C. than from the hemoglobin heated at 80°C. Actually, as will be shown in the following paper, the yield is the same, about 30 per cent in both cases.

The preparation of the coagulated protein is carried out as follows:

To 5 cc. of a 14 per cent hemoglobin solution are added 7.25 cc. H_2O and 4.75 cc. $\frac{N}{5}$ HCl (just enough to keep the denatured hemoglobin in solution). The

solution is heated at 80°C. for 3½ minutes, and while still hot a mixture of 5 cc. $\frac{M}{5}$ KH_2PO_4 and 7.25 cc. $\frac{N}{5}$ NaOH is added to it and mixed at 80°C. The filtrate from this coagulum is clear and colorless. When it is heated to over 90°C. it shows practically no opalescence, indicating that coagulation is complete. Similarly the hemoglobin heated at 100°C. is completely precipitated on neutralization.

(*4*) *Urea.* We find that concentrated solutions of urea denature proteins and keep the denatured proteins in solution. If hemoglobin is allowed to stand in urea solution, then on removal of the urea by dialysis or on dilution of the solution the protein precipitates completely. Furthermore, as will be shown in a later paper, denaturation by urea can be followed by viscosity measurements. As denaturation proceeds, the solution becomes more viscous. Finally, when all the protein has been converted into a form which is insoluble in water when the urea is removed, the viscosity remains constant at a high level. Were denaturation as tested for by solubility incomplete one would expect a further change of viscosity with time.

The urea coagulum is prepared as follows:

200 grams of urea are dissolved in 250 cc. of a 14 per cent hemoglobin solution and allowed to stand for 48 hours at room temperature. The solution is added slowly and with stirring to about 4 liters of water. A flocculent precipitate appears. This is thoroughly washed with water by decantation. The coagulum is concentrated by centrifuging at low speed.

II. Hemoglobin as a Typical Coagulable Protein

In order that experiments with completely denatured hemoglobin may be of general significance in the study of coagulation it must be made clear that what we have called the denaturation of hemoglobin is the counterpart of the denaturation of other proteins, not a peculiarity of hemoglobin. We shall accordingly show in detail that all the procedures such as heating which convert egg albumin into an insoluble denatured form likewise convert hemoglobin into an insoluble form, and that the insoluble hemoglobin has besides insolubility in water the other characteristics of denatured protein. In making this comparison it has been necessary in some cases to provide the data for hemoglobin. On the other hand, where experiments on the denaturation of hemoglobin have led to the discovery of new phenomena,

these same phenomena have been found to exist in the denaturation of other proteins.

Hitherto in the investigation of some hemoglobin derivatives attention has been focussed on the non-protein part of the molecule and the protein part has been ignored. In such studies a denatured form of hemoglobin has been almost unknown. We now know (2) however, that the preparation of some hemoglobin derivatives, such as hemochromogen, involves the denaturation of the protein.

(*1*) *Heat.* Hemoglobin like egg albumin can be coagulated by heat. In both cases the denaturation proceeds as a monomolecular reaction with the striking and characteristic temperature coefficient of over 600 for 10° (9). The rate of most chemical reactions is increased from 2 to 4 times for a rise in temperature of 10°.

At first sight it might seem that the temperature coefficient of the denaturation of hemoglobin is different from that of other proteins, for although the coefficient for methemoglobin is over 600, for oxyhemoglobin it is only 13.8 and for carbon monoxide hemoglobin 5.2 (9). But when oxy or carbon monoxide hemoglobin is heated two entirely different reactions take place, the hemoglobin is converted into methemoglobin (that is, the iron atoms of the native protein are oxidized) and the protein is denatured. In order, therefore, to study heat denaturation alone apart from methemoglobin formation, it is necessary to start with methemoglobin.

Both dry hemoglobin and dry egg albumin can be heated to 100° without the protein losing its solubility in water.

(*2*) *Acid and Alkali.* If acid or alkali is added to either hemoglobin or egg albumin and the solution is subsequently brought to the isoelectric point of the protein, the protein is precipitated. Acid and alkali thus denature both hemoglobin and egg albumin.

(*3*) *Alcohol* coagulates both hemoglobin and egg albumin.

(*4*) *Ultra-violet light* coagulates both hemoglobin and egg albumin (8).

(*5*) *Shaking.* It is known (11) that egg albumin can be completely coagulated by shaking. We have found that the same holds true for hemoglobin.* As in the case of heat coagulation, it is desirable to start the experiments with methemoglobin.

* Since this section was written Wu and Ling (16) have published an account of the coagulation of hemoglobin by shaking.

If a solution of methemoglobin (protected from bacterial reduction by a little toluol) is shaken, coagulated hemoglobin gradually appears. If more toluol is added and an emulsion with a large surface is formed, much more protein is coagulated. For example, a 10 per cent solution of salt-free methemoglobin is shaken for 16 hours with an equal volume of toluol. All the hemoglobin is coagulated. The supernatant fluid after centrifugation of the precipitate is clear and colorless. As a control experiment a similar methemoglobin solution containing only enough toluol to saturate the solution is allowed to stand 16 hours. Very little coagulum is formed. This makes it improbable that toluol itself coagulates hemoglobin.

On long standing methemoglobin gradually coagulates, as egg albumin does, even without shaking. It appears then that both hemoglobin and egg albumin coagulate at surfaces and that shaking merely accentuates this property.

(6) *Thiocyanate, Iodide, and Salicylate.* We have found that dilute solutions of these salts denature hemoglobin. Von Fürth (6) found that 5 per cent solutions of the same salts coagulate myogen. So thiocyanate, iodide, and salicylate probably denature proteins in general.

The experiments are carried out as follows:

1/10 cc. of salt-free methemoglobin (about a 10 per cent solution) is added to 5 cc. of a solution containing KI, KCNS or Na salicylate. The salts are present in a concentration of 0.6 M. In one series these salts are in an acetate solution buffered at pH 5.2 and in another series they are in a borate solution of pH 9.2. The acid tubes all contain precipitates after 12 hours. A pigment in the alkaline tubes is still in solution and no precipitate is detectable.

A similarity between this method of denaturation and others may be tested when the alkaline solution containing any one of these three salts is reduced with sodium hydrosulfite; it then has the absorption spectrum of hemochromogen, the substance formed when methemoglobin is reduced and denatured—whether by heat, acids, alkalies, alcohol, ultra-violet light or shaking. It is therefore probable that methemoglobin solutions at pH 9.2 are also denatured by the KI, KCNS, and Na salicylate.

These solutions (without addition of sodium hydrosulfite) are now

placed in collodion tubes and dialyzed against distilled water as the outer liquid. As dialysis proceeds, a brown precipitate appears in the collodion tubes. This precipitate has the same properties as the coagulum formed from methemoglobin by the action of the various agents we have studied. We therefore conclude that methemoglobin has been denatured by 0.6 M solutions of these salts.*

(7) *Urea.* As described in the first part of this paper, concentrated urea solutions denature hemoglobin and keep denatured hemoglobin in solution. When the urea solution is diluted; the protein precipitates completely and can be washed free of urea. Apart from the precipitation of the protein on dilution of the urea, there is spectroscopic evidence that the hemoglobin has been denatured. If the reducer, $Na_2S_2O_4$, is added, the spectrum of hemochromogen, that is denatured hemoglobin, can be observed.

We find that urea has the same effect on egg and serum albumin that it has on hemoglobin. A concentrated urea solution is added to some egg albumin and the solution is allowed to stand for a day. When a few drops are added to about 15 cc. of water buffered at a pH of 4.7 (isoelectric point of egg albumin) a voluminous protein precipitate occurs. The protein redissolves when urea crystals are dissolved in the water. Apparently urea can denature egg albumin and likewise dissolve the denatured protein.

If the same experiment (with urea) is repeated with serum albumin it is found that no precipitate appears when several times as much water is added as in the preceding experiment. The solution is placed in a collodion tube and dialyzed overnight against running distilled water. Next morning the protein is found precipitated at the bottom of the tube. It would seem as if urea denatures serum albumin and also dissolves denatured serum albumin, but a less concentrated urea solution is needed to prevent the precipitation of denatured serum albumin than is needed in the case of denatured hemoglobin or egg albumin.

It has been shown that heat, acid, alkali, alcohol, ultra-violet light, KCNS, KI, Na salicylate and urea all convert hemoglobin as they do egg albumin into an insoluble form, and that the kinetics of dena-

* Other experiments show that much more dilute solutions of these salts suffice to denature hemoglobin.

turation is the same in the cases of hemoglobin and egg albumin. In order to complete the evidence that hemoglobin is a typical coagulable protein, it remains to show that the insoluble protein formed from hemoglobin by heat, etc., has in addition to insolubility in water all the other characteristic properties of denatured protein.

(*1*) *Solubility.* Both denatured hemoglobin and denatured egg albumin after being precipitated at their isoelectric points can be dissolved again in acid or alkali. Even at its isoelectric point denatured hemoglobin can be dissolved not only in concentrated urea solution, as has already been pointed out, but also in concentrated solutions of KCNS, KI, and Na salicylate. A urea solution not concentrated enough to dissolve isoelectric coagulated hemoglobin, may still suffice to dissolve the coagulum if the solution is slightly acid or alkaline, so slightly acid or alkaline that without the urea, very little of the protein would dissolve. Denatured egg and serum albumins are likewise soluble in solutions of urea and of the salts mentioned. As Spiro (14) noticed, serum albumin can be heated in saturated urea solution without any visible precipitate being formed.

(*2*) *Viscosity.* We have found that when either hemoglobin or egg albumin is denatured there is an increase in viscosity characteristic of denaturation. This change is of a greater order of magnitude than the changes associated with the ionization of the soluble proteins, which are the concern of most investigation of the viscosity of protein solutions. Our experiments on the viscosity of solutions of denatured proteins will be published in a separate paper.

(*3*) *Species Specificity.* When proteins are denatured, their species specificity, as determined immunologically, is decreased. No similar immunological experiments have been done with denatured hemoglobins. But, as we have shown in previous papers (1, 2) it is impossible to distinguish the various denatured hemoglobins by spectroscopic or gas affinity measurements, although by these same methods it is easy to distinguish native hemoglobins of even the most closely related species.

SUMMARY

As a preliminary to the study of the reversal of the coagulation of hemoglobin several methods are described for the preparation of

completely denatured and coagulated hemoglobin and the evidence is given that hemoglobin is a typical coagulable protein.

BIBLIOGRAPHY

1. Anson, M. L., and Mirsky, A. E., *J. Physiol.*, 1925, 60, 50.
2. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1925, 9, 169.
3. Anson, M. L., and Mirsky, A. E., *J. Biol. Chem.*, 1927, 74, p. lvii.
4. Chick, H., and Martin, C. J., *J. Physiol.*, 1910, 40, 404.
5. Chick, H., and Martin, C. J., *J. Physiol.*, 1911, 43, 1.
6. von Fürth, O., *Arch. Exp. Path.*, 1896, 37, 390.
7. Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1899–1900, 66, 110.
8. Hasselbalch, K. A., *Biochem. Z.*, 1909, 19, 435.
9. Hartridge, H., *J. Physiol.*, 1912, 44, 34.
10. Heidelberger, M., *J. Biol. Chem.*, 1922, 53, 31.
11. Ramsden, W., *Proc. Roy. Soc. London*, 1903–1904, 72, 156.
12. Sørensen, S. P. L., *Comp. rend. Trav. Lab. Carlsberg*, 1925, 15, 1.
13. Spiegel-Adolf, M., *Biochem. Z.*, 1926, 170, 126.
14. Spiro, K., *Z. Physiol. Chem.*, 1900, 30, 182.
15. Wu, H., and Lin, K. H., *Chinese J. Physiol.*, 1927, 1, 219.
16. Wu, H., and Ling, S. M., *Chinese J. Physiol.*, 1927, 1, 431.

PROTEIN COAGULATION AND ITS REVERSAL

The Reversal of the Coagulation of Hemoglobin

By A. E. MIRSKY and M. L. ANSON

(From the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y., and the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, June 27, 1929)

The coagulation of hemoglobin takes place in two steps (7, 5, 6). The first step is denaturation, a chemical change in the protein which makes it insoluble around its isoelectric point. The second step is merely the precipitation of the insoluble denatured protein. It has hitherto been believed that denaturation is irreversible.

The preceding paper (4) has already described the preparation of completely coagulated hemoglobin and has given the evidence that the denaturation and coagulation of hemoglobin are in all known respects like the denaturation and coagulation of other proteins such as egg albumin. The first part of this paper describes the preparation from completely coagulated hemoglobin of soluble, apparently native hemoglobin. The second part gives the evidence that this soluble protein is the same as the original native hemoglobin which had never been modified. From these experiments the conclusion is drawn that the coagulation of hemoglobin is reversible. Since hemoglobin is a typical coagulable protein, if the coagulation of hemoglobin is reversible then the coagulation of proteins in general is probably reversible. The coagulation of other proteins, in particular globin and the tissue proteins, and the biological significance of coagulation will be discussed in later papers.

Part I. The Preparation of Soluble Hemoglobin from Coagulated Hemoglobin

If an acid or alkaline solution of completely coagulated hemoglobin is neutralized all the protein is precipitated. If, however, before com-

plete neutralization the solution of denatured hemoglobin is allowed to stand in slightly alkaline solution under conditions to be described, then on complete neutralization only a part of the protein is precipitated. The remaining soluble part is the apparently reversed hemoglobin. How much of this soluble apparently reversed hemoglobin is obtained depends, other factors being constant, on the state of the prosthetic group, heme, and on the species of the hemoglobin.

In the case of the ox hemoglobin, if the heme is in the oxidized state, very little soluble hemoglobin is obtained from the coagulum. If a little of the reducer, sodium hydrosulfite, is added, much more is obtained. And if cyanide is added still more is obtained.

In the case of horse hemoglobin, practically no soluble protein is obtained from the coagulum whether the heme is either oxidized or reduced. When cyanide is used, however, about 30 per cent of the denatured protein yields soluble crystalline apparently native horse hemoglobin. Because it can be crystallized most of the experiments have been done with horse hemoglobin.

The results with ox hemoglobin show that cyanide is not essential for the apparent reversal of coagulation. And even though cyanide appears to be necessary for an adequate yield with horse hemoglobin, neither the initial soluble nor the final soluble product is combined with cyanide. So the question of the function of the cyanide is separate from the question of whether denaturation is reversible.

The experiments with horse hemoglobin show clearly that the mere fact that denaturation cannot be reversed under a given set of conditions does not mean that denaturation cannot be apparently reversed under other more suitable conditions.

It is to be expected that the state of the prosthetic group and the species of the hemoglobin should influence the ease of the apparent reversal of denaturation because these same factors are known to influence the ease of denaturation itself. Some hemoglobins are more readily denatured than others and methemoglobin is more readily denatured than oxy or carbon monoxide hemoglobin. That the heme part of the hemoglobin molecule should modify its protein properties is not surprising. We have already shown (2) that the globin part of the molecule modifies greatly the properties of the non-protein heme part, in particular its reactions with oxygen.

The mechanism of the effect on apparent reversal of the reducer, $Na_2S_2O_4$ and of cyanide is not yet understood. It is not even certain that $Na_2S_2O_4$ and CN act solely on the non-protein part of hemoglobin. It might be desirable, nevertheless, to recall the reactions of cyanide with the various hemoglobin derivatives. Cyanide combines with both oxidized and reduced heme when the heme is joined to denatured globin but only with oxidized heme when the heme is joined to native globin. In other words, cyanide combines with hematin, hemochromogen and methemoglobin but not with oxy, carbon monoxide or reduced hemoglobin.

One step in the procedure for obtaining apparently reversed horse hemoglobin consists in dissolving the coagulated protein in a slightly alkaline solution containing cyanide. It is desirable to buffer the solution in order to protect the protein which is sensitive to alkali. The dissociation constant of hydrocyanic acid is 72×10^{-10}, about the same as that of boric acid. Mixtures of NaCN and HCl may accordingly be used as buffers in the region around pH 9.14. Cyanide, then, is used in these experiments for two entirely different purposes, to facilitate apparent reversal by reacting with denatured hemoglobin and as part of a buffer mixture to maintain a suitable hydrogen ion concentration.

Technique for Apparent Reversal of Coagulation

Heat Coagulated Ox Hemoglobin. A hemoglobin solution is prepared from ox blood by laking the washed corpuscles with ether. To 1 cc. of the hemoglobin solution are added 8 cc. of water and 1 cc. $\frac{N}{5}$ HCl; the solution is then heated at 80°C. for 3½ minutes. While at 80°C. a mixture of 3 cc. $\frac{M}{5}$ KH_2PO_4 and 2½ cc. $\frac{N}{5}$ NaOH is added so as to bring the pH to 6.8. The suspension is cooled and then filtered. The filtrate is clear and colorless. When boiled it turns only faintly opalescent indicating that practically all the hemoglobin has been coagulated.

In order to obtain the apparent reversal of coagulation the denaturation experiment is repeated but instead of adding the phosphate buffer the solution is allowed to cool and 10 cc. of $\frac{N}{25}$ NaOH are added making it faintly alkaline. A little $Na_2S_2O_4$ is added and the mixture allowed to stand for 2 to 3 minutes. With a spectroscope the absorption bands of both hemochromogen and reduced hemo-

globin are visible. The suspension is filtered and the filtrate gently shaken in the air to oxygenate. The absorption bands of the soluble, apparently native HbO_2 can be distinctly seen. If the heating is at the boiling point (20° higher) substantially the same result is obtained. The only difference is that now the absorption bands of the soluble oxyhemoglobin are a little fainter. If this soluble HbO_2 were merely some native protein that had not been coagulated, hundreds of times as much HbO_2 would be obtained from the solution heated at 80°C. as from that at 100°C.

In the case of horse hemoglobin the denatured protein is first dissolved in acid. Alkali causes secondary changes in the protein beyond mere denaturation. The acid solution is brought to about pH 9 or slightly more alkaline with a KCN-HCl buffer. The solution is then neutralized and the resulting precipitate filtered off. The filtrate is saturated with CO and reduced with $Na_2S_2O_4$. Finally ammonium sulfate is added. The amorphous precipitate first found settles to the bottom. The pigment in the clear supernatant fluid gradually separates out completely in the form of crystalline apparently native carbon monoxide hemoglobin. Carbon monoxide is used because carbon monoxide hemoglobin is more stable than oxyhemoglobin. Oxyhemoglobin together with methemoglobin is obtained if the carbon monoxide is not used and the reducer is oxidized.

The cyanide buffer solution is made by adding to 1 gm. of KCN dissolved in 4.16 cc. water 23.13 $\frac{N}{5}$ HCl. The solution is blue to thymol blue.

Preparation of Hemoglobin. Red corpuscles washed four times with isotonic salt solution are shaken up with an equal volume of water and a fifth the total volume of toluol (8). After the solution has stood overnight in the cold, the toluol and stromata are removed by filtration or centrifugation.

Heat Coagulated Horse Hemoglobin. A tube containing 7 cc. of 10.85 per cent of horse hemoglobin and 10 cc. of water is placed in boiling water for 3½ minutes. The temperature rises to 80°C. in about half a minute and then gradually goes up to a little over 90°C. The contents are not agitated, thus facilitating subsequent solution of the coagulum. The tube is placed in cold water for a few minutes. 4 cc. $\frac{N}{5}$ HCl are poured in (down the side of the tube) without stirring. The contents of the tube are mixed gently as the coagulum dissolves and then poured into a 50 cc. flask. 4 cc. $\frac{N}{5}$ NaOH and 2.5 cc. of the cyanide buffer solution are mixed and added to the flask making its contents faintly alkaline. A brownish red precipitate is observed. The mixture is allowed to stand for 2 to 6 hours. Most of the precipitate gradually redissolves. 5 cc. $\frac{M}{5}$ KH_2PO_4 are added to bring the mixture nearer the neutral point, to a pH of about 7 to 7.4. A brownish red precipitate is deposited. The suspension is filtered or centrifuged and a deep red solution obtained. Some carbon monoxide is bubbled

through this and immediately a knife point of solid $Na_2S_2O_4$ is added to reduce the solution. The flask is stoppered, gently shaken and then allowed to stand in the dark for about a half hour. To the solution is added about one and one-half times its volume of saturated $(NH_4)_2SO_4$. The mixture is allowed to stand in the dark for 24 to 48 hours. A flocculent, amorphous precipitate gradually settles out and is discarded. The clear, supernatant fluid is poured into a tall vessel. Through this fluid a little carbon monoxide is bubbled. The vessel is stoppered and allowed to stand in the dark. In the course of a few days large crystals gradually appear on the walls and bottom of the flask. At the end of a month, if not before, practically all of the color has left the solution and there is a large deposit of crystals. There is practically no amorphous matter mixed with the crystals. Some of them are so large that their form can be observed with the unaided eye. They can be washed with a little of the $(NH_4)_2SO_4$ solution. These crystals constitute the soluble apparently reversed protein.

Horse Hemoglobin Denatured by Acid. 6.5 cc. of the 10.85 per cent horse hemoglobin solution are mixed with 10 cc. $\frac{N}{5}$ HCl and the mixture kept at 40°C. for 3 minutes. After cooling a mixture of 2.5 cc. of the cyanide solution used above to dissolve the heat coagulum and 10 cc. $\frac{N}{5}$ NaOH are added to the denatured hemoglobin. From this point the procedure is exactly the same as that described above. In some of our experiments before obtaining an alkaline solution of denatured protein the acid solution was carefully neutralized, so that the denatured protein precipitated. This precipitate was thoroughly washed with distilled water not only until the washings were colorless, but even until they gave no test for chloride. The precipitate was then dissolved in alkali.

Horse Hemoglobin Denatured in Acid at Two Different Temperatures. The same yield of apparently reversed hemoglobin is obtained whether the protein is heated in acid at 80°C. or 100°C. The rate of denaturation of oxyhemoglobin is more than a hundred times faster at the higher than at the lower temperature. Did the soluble protein come from protein which had never been denatured then one ought to get much less soluble protein from the protein heated to 100°C. than from the protein heated to 80°C. Since the same yield is obtained in both cases the final soluble protein is probably not merely native protein which had never been denatured. Just enough acid is used to prevent visible precipitation. Boiling a neutral suspension of coagulated hemoglobin causes the protein to form hard clumps which are hard to dissolve. If the acid is neutralized immediately after the heating all the protein precipitates indicating complete denaturation.

To 5 cc. of 13.8 per cent hemoglobin are added 7.25 cc. water and 4.75 cc. $\frac{N}{5}$ HCl. The solution is heated for 3½ minutes. After cooling, a mixture of 4.75 $\frac{N}{5}$ NaOH and 2.5 cc. cyanide buffer is added. The rest of the procedure is as already described.

Hemoglobin Denatured by Urea. About 15 cc. of the suspension of protein denatured by urea prepared as described in the preceding paper are dissolved by the addition of 4 cc. $\frac{N}{5}$ HCl, and the procedure is then completed in the usual manner. The crystals obtained are less perfect than those obtained in the other cases, and the yield is smaller.

The Yield. The yield in the experiments described in this paper was determined colorimetrically, using carbon monoxide hemoglobin in ammonium sulfate solution as a standard. The solutions were taken for estimation after the addition of ammonium sulfate, after the separation of the amorphous precipitate, but before the separation of crystals. A part of the solution was always kept to make sure that all the pigment would eventually separate out in crystalline form. The solutions contained no pigment other than carbon monoxide hemoglobin which could be detected spectroscopically and they matched in color the standard solutions.

In the case of the horse hemoglobin denatured by heating, by acid or by heating in acid, the yield of apparently native carbon monoxide hemoglobin is about 30 per cent of the total original hemoglobin. Some of the remaining 70 per cent was of course lost in purely mechanical ways.

We are now investigating the factors influencing the yield and the yields obtained when one starts with the "reversed" and the "non-reversed" fractions.

Part II. Comparison of Apparently Reversed and Native Horse Hemoglobin

The chemical changes in the apparent reversal of denaturation and coagulation are as obscure as the changes in denaturation itself. In order, however, to prove the possibility of reversal it is not essential to investigate or understand the mechanism of any intermediate reactions. It is necessary to examine only the initial and final prod-

ucts, to show that one starts with coagulated protein and that one finally obtains from it native protein.

The preceding paper has given the evidence that the initial product was completely denatured. It remains to examine whether the soluble apparently reversed horse hemoglobin finally obtained from completely coagulated horse hemoglobin has not only all the general characteristics of native hemoglobin but quantitatively the precise properties of native horse hemoglobin. The apparently reversed and the original native proteins have accordingly been compared qualitatively in respect to solubility,[1] and quantitatively in respect to coagulation, crystal form, color, spectrum, reactions with oxygen and carbon monoxide, and species specificity. No differences have been found.

The advantages of using hemoglobin, a conjugated protein, are now apparent, for due to the union of protein to the iron-pyrrol complex, the molecule possesses in addition to all the ordinary protein properties, others open to exact measurement that simple proteins do not possess. Since practically all of the properties of the iron-pyrrol complex in hemoglobin are affected by the state of the globin, changes in the protein are reflected in the clearly defined behavior of the non-protein part of the molecule.

It is possible that there are differences between the apparently reversed and the original native proteins which have not been detected. A deeper knowledge than we now possess of denaturation and reactions resulting in apparent reversal may make such differences significant. For the present, however, the most reasonable conclusion from the experiments described in this paper is that the denaturation and coagulation of hemoglobin, and hence probably of the other coagulable proteins, are indeed reversible.

The Crystals. A denatured protein has never been crystallized, but the soluble hemoglobin obtained from coagulated hemoglobin is readily crystallizable. In the present experiments the protein is crystallized by salting out slowly with $(NH_4)_2SO_4$. The less concentrated the hemoglobin solution the more salt is needed; on the other hand, the less salt used the slower the crystallization, and consequently

[1] Quantitative solubility measurements are now being carried out.

the larger and more perfectly formed the crystals. The salt concentration suitable for crystallizing apparently reversed protein is the same as for crystallizing native carbon monoxide hemoglobin from a protein solution of the same concentration. The crystals obtained (Fig. 1) are of the same crystal form and habit as the crystals of native horse HbCO. Most of the further examination of the apparently reversed protein is of a solution of these crystals.

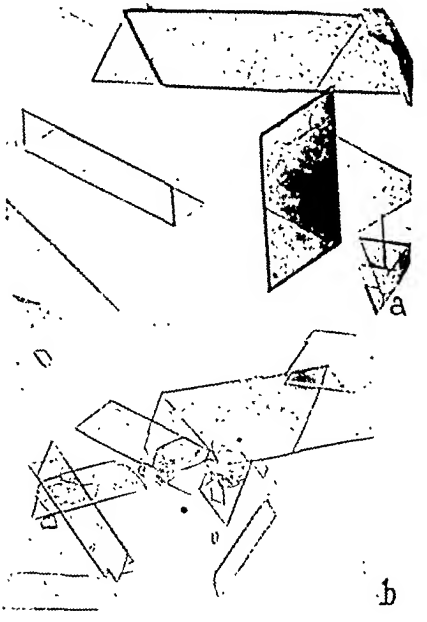


FIG. 1 *a* and *b*. (*a*) crystals of carbon monoxide hemoglobin; (*b*) crystals of "reversed" carbon monoxide hemoglobin.

Since the apparently reversed hemoglobin can be crystallized and by a procedure not described in this paper crystallized completely in a few minutes, the apparently reversed hemoglobin is a definite pure substance and not merely a vague disintegration product of denatured hemoglobin.

Solubility. Denatured hemoglobin is practically insoluble in water at its isoelectric point, about pH 6.8. It does not even color the water. The crystals, however, are readily soluble in a pH 6.8 phosphate buffer. A deep red solution containing 5 per cent of apparently native HbCO is easily prepared.

Coagulation. The solution of apparently reversed protein can be coagulated by heat. The temperature of coagulation is a highly characteristic constant of a protein. It is, however, affected by salt. If apparently reversed protein dissolved in a $(NH_4)_2SO_4$ solution is compared with a solution of native HbCO of the same concentration in the same salt solution by placing the tubes containing them side by side in boiling water, coagulation occurs at the same instant in both solutions. The temperature of coagulation of the apparently reversed protein is therefore the same as that of the native protein from which it is prepared. The apparently reversed protein can be denatured by acid, alkali, etc.

Color. The color of the apparently reversed protein matches that of ordinary horse HbCO, when they are compared in the colorimeter. It has already been stated that when $(NH_4)_2SO_4$ is added to the solution of apparently reversed protein an amorphous precipitate settles out before crystallization occurs. This precipitate is discarded. If, however, the color of the solution is examined before the removal of this substance, the color is distinctly different from that of uncoagulated protein, showing that with the colorimeter it is possible to detect a small amount of foreign pigment. The apparent coagulation temperature of this unpurified preparation may be more than 20° lower than that of original native protein.

Absorption Bands. The absorption spectrum of the apparently reversed protein is indistinguishable from that of native hemoglobin. With the Hartridge reversion spectroscope the positions of the absorption bands can be measured to about 2 Å. u., but we have been unable to detect any difference in the patterns of the absorption spectra or the positions of the absorption bands of the HbO_2, HbCO, methemoglobin, or sulfhemoglobin obtained from the apparently reversed and the original native protein.

Combination with Oxygen. Denatured hemoglobin does not form that loose combination with oxygen so characteristic of hemoglobin. The apparently reversed hemoglobin combines loosely with oxygen and can be oxygenated and reduced repeatedly.

Gas Affinities. Hemoglobin combines loosely with both oxygen and carbon monoxide. If hemoglobin is exposed to a mixture of the two gases at equilibrium, part of the pigment is combined with oxygen

and part with carbon monoxide. The reaction obeys the mass law equation.

$$\frac{[HbCO]\,[O_2]}{[HbO_2]\,[CO]} = K$$

K, the equilibrium constant, is a measure of the relative affinities of hemoglobin for the two gases and is different for each species of hemoglobin. Barcroft, Oinuma and the writers (1) showed that K can be predicted from purely spectroscopic measurements, being a function of the number of Ångström units separating the α bands of oxy and carbon monoxide hemoglobins. Since these bands are in the same positions in the apparently reversed hemoglobin as in ordinary native hemoglobin, one would expect the K's to be the same in both cases, too. This we have found to be the fact.[2] The technique has already been described (1). To make sure that equilibrium was reached, the equilibrium was approached from both sides.

In developing the method for determining K it was found possible to estimate the relative quantities of HbO_2 and HbCO in a mixture of both by measuring the position of the α absorption band of the mixture. The position of this band is a function of the composition of the mixture. This function is exactly the same for HbO_2 and HbCO of apparently reversed hemoglobin as it is for original hemoglobin. In one more way, therefore, the spectroscopic properties of apparently reversed hemoglobin are quantitatively the same as those of native hemoglobin.

Species Characteristics. By means of crystal form and by spectroscopic and gas affinity measurements (1) the hemoglobins of different species are easily distinguished. The soluble, apparently native hemoglobin prepared from coagulated horse hemoglobin even by these sensitive tests cannot be distinguished not only from native hemoglobin in general but from native horse hemoglobin in particular. This is especially interesting because, as we have already shown (2, 3) denatured hemoglobins of various species cannot be distinguished from each other by spectroscopic or gas affinity measurements. After apparent reversal of coagulation the species characteristics once more become observable.

[2] This experiment was performed only once, though in duplicate, so a repetition would be desirable.

SUMMARY

1. The preparation from completely coagulated hemoglobin of crystalline soluble hemoglobin is described.

2. This soluble hemoglobin by all the tests tried has been indistinguishable from normal native hemoglobin which has never been coagulated.

3. The coagulation of hemoglobin is probably reversible.

4. Since hemoglobin is a typical coagulable protein, protein coagulation in general is probably reversible.

BIBLIOGRAPHY

1. Anson, M. L., Barcroft, J., Mirsky, A. E., and Oinuma, S., *Proc. Roy. Soc. London, Series B.*, 1924, 97, 61.
2. Anson, M. L., and Mirsky, A. E., *J. Physiol.*, 1925, 60, 50.
3. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1925, 9, 169.
4. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1929, 13, 12.
5. Chick, H., and Martin, C. J., *J. Physiol.*, 1910, 40, 404.
6. Chick, H., and Martin, C. J., *J. Physiol.*, 1911, 43, 1.
7. Hardy, W. B., *Proc. Roy. Soc. London, Series B.*, 1899–1900, 66, 110.
8. Heidelberger, M., *J. Biol. Chem.*, 1922, 52, 31.

THE PREPARATION OF RELATIVELY PURE BACTERIOPHAGE

By A. P. KRUEGER and H. T. TAMADA

(From the Department of Bacteriology and Experimental Pathology, Stanford University, California)

(Accepted for publication, June 29, 1929)

Studies on the nature of the bacteriophage are usually carried out with lysates which contain, in addition to the lytic principle itself, the nutrient constituents of the broth and substances set free from the lysed bacteria. While such preparations are suitable for many types of investigation, there are certain problems, *e.g.*, those dealing with the physical or chemical properties of the bacteriophage, which require the use of relatively pure phage.

That the composition of the medium in which bacteriophage is suspended exerts considerable influence on the physical state of the lytic agent was brought out by recent work of the authors (1), who showed that purified bacteriophage free from bacterial proteins not only has a smaller particle size than non-purified phage but also will readily unite with proteins or protein derivatives to form the larger particles found in ordinary lysates. This point, that the lytic agent is commonly associated with colloidal aggregates has been repeatedly stressed by Bronfenbrenner (2) and it appears quite logical that physical-chemical experiments dealing with the phage itself should either be performed with purified bacteriophage free from the influence of a complex suspending medium such as nutrient broth, or else that the influence of the menstruum must be taken into account.

Arnold and Weiss (3) devised a means of preparing bacteriophage free from bacterial proteins based upon the diffusion of lytic corpuscles from a lysogenic culture seeded on nutrient agar into a bottom layer of plain agar. Several extractions are made from the latter stratum and one finally obtains a clear uncolored phage suspension which gives a slight positive reaction with the usual protein reagents. This may be further purified by precipitating the small residual protein

fraction with 14 per cent sodium sulfate. Such preparations are free from antigenic bacterial proteins as evidenced by their failure to produce antibodies other than antilysins when injected into animals.

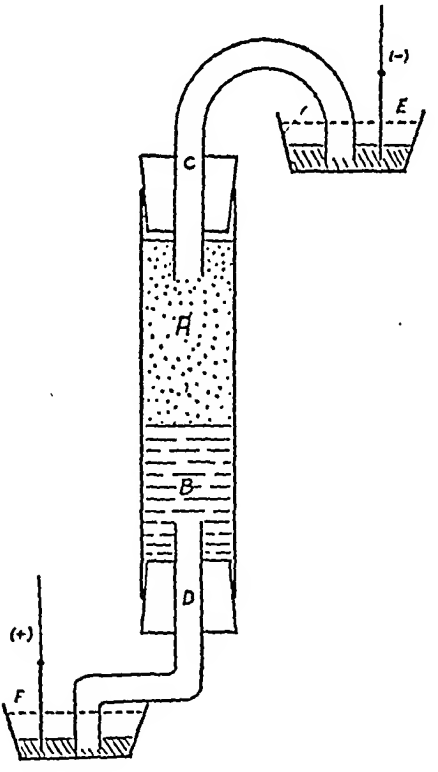


FIG. 1. Apparatus for preparation of relatively pure bacteriophage. *A*, bacteriophage suspension. *B*, gel of 0.5 per cent purified agar and 0.2 per cent C. P. NaCl in distilled water. *C* and *D*, glass bridges containing 3.0 per cent pure agar gel with 0.5 per cent C.P. NaCl. *E*, porcelain cup with crystals of $CuCl_2$ at bottom. Water to level of dotted line. Negative pole (Copper) of D.C. circuit dips into crystals. *F*, porcelain cup with crystals of NaCl at bottom. Water to level of dotted line. Silver foil attached to positive pole of D.C. circuit dips into crystals.

This procedure has the disadvantage, however, that during the numerous extractions required to remove the proteins much of the phage is removed as well and in our hands the method has not yielded

protein-free suspensions of high lytic titre. The most active preparation we were able to make following Arnold and Weiss' process was not effective beyond a dilution of 10^{-5}.* In addition the concentration of sodium sulfate in the final product is objectionable for certain types of work.

Investigations we had under way demanded the use of strongly lytic suspensions containing a minimum concentration of the other materials found in ordinary lysates. Since it is known that the bacteriophage carries a negative charge from pH 3.6 to pH 7.6 (Todd) (4) it seemed quite reasonable to attempt its concentration at the anode of some sort of cataphoresis apparatus, employing, if possible, a pH lower than the isoelectric ranges of the other constituents of the lysate in order to retain them at the cathode. After numerous experiments with various forms of apparatus the following procedure was developed and has proven entirely satisfactory.

Method of Preparing Relatively Pure Bacteriophage Suspensions

Pure agar is prepared according to the method of Dominikiewicz (5). To a 0.5 per cent suspension of the pure agar in distilled water 0.2 per cent C.P. NaCl is added and the mixture is sterilized by boiling or autoclaving.

The apparatus is explained in the accompanying diagram and requires no special mention except to note that convenient dimensions for the tube are 15 cm. by 2.5 cm. It is our practice to sterilize the assembled apparatus and to then fill the bridges with sterile purified 3 per cent agar containing 0.2 per cent sodium chloride. This is readily done by pipetting the agar into the inverted upper bridge and by resting the distal end of the lower bridge against a flat surface while it is being filled. When the agar is hardened the lower bridge and stopper are fitted in place. 20 cc. of the 0.5 per cent agar containing 0.2 per cent sodium chloride is poured into the cylinder observing sterile precautions. After the agar is hard 30–40 cc. of ordinary phage suspension is layered over it and the upper stopper placed in position.

A current of 100–125 volts and from 5–12 milliamperes is passed through the apparatus for 18 to 20 hours (this need not be continuous). The phage above the agar is best changed once or twice during the run. At the end of this period the broth is taken up by pipette, discarded, and the upper surface of the agar washed

* We have employed the technic recommended by d'Herelle (The bacteriophage and its behavior, Williams and Wilkins, 1926, 96) in all quantitative estimations of the lytic particles.

with several changes of sterile physiological saline solution. The lower bridge and stopper are removed and the agar allowed to slide out gradually. It is sliced into thin sections with a sterile spatula as it issues forth and is received in a sterile Petri dish, discarding the layer 0.5 cm. thick nearest the broth. The remainder is thoroughly macerated for 2–3 hours with 10–30 cc. of sterile water or saline solution, depending upon the type of suspension and the concentration desired. It is then freed from agar particles by filtration through a coarse Chamberland candle or by centrifuging.

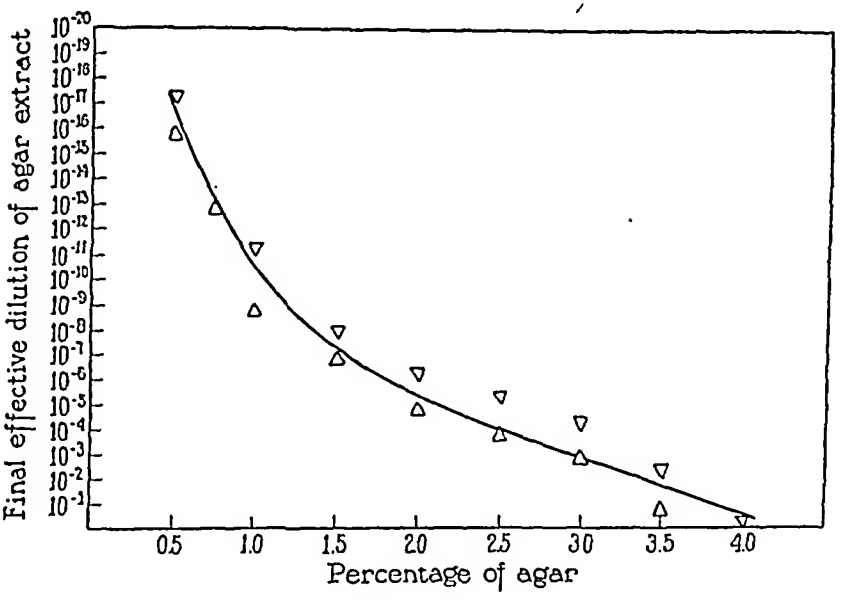


CHART 1. Effect of agar concentration on final phage content of extract. For each experiment: E.M.F. 100–102 V. 7–10 milliamperes. Current on 18 hours. 20 cc. agar of varying percentages from 0.5 per cent to 4 per cent all containing 0.2 per cent C.P. NaCl. Extractive: 20 cc. physiological saline. Anti-*coli* bacteriophage. Broth pH 7.4.

DISCUSSION

It was at first thought that the phage suspension used in the apparatus should be adjusted to a relatively low pH on the acid side of the isoelectric points of the proteins and their derivatives so that these substances would carry a positive charge and would therefore concentrate at the cathode. However, experiments run with ordinary Martin's broth suspensions (pH 7.0–7.4) constantly resulted in the

precipitation at the cathode of a dense coagulum giving the usual qualitative protein reactions. Furthermore, there was no migration of these compounds into the agar. Consequently, any such adjustment of pH was found unnecessary for our present purposes.

Early experiences indicated that the percentage of agar employed as well as the length of time during which a given E. M. F. is allowed to act markedly influenced the lytic titre of the extract. Two sets of experiments were accordingly undertaken to determine the optimal

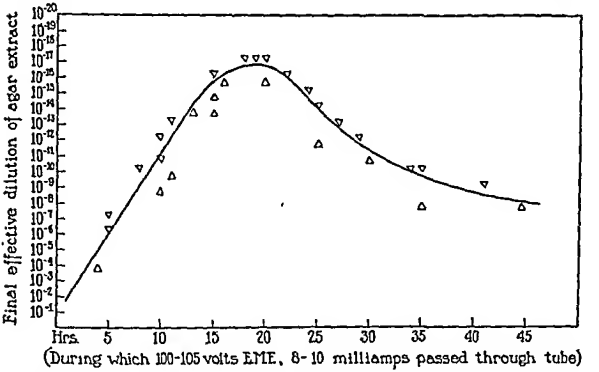


CHART 2. Effect of varying time of current passage on final phage content of extract. For each experiment: E.M.F. 100–105 V. 8–10 milliamperes. 20 cc. 0.5 per cent agar with 0.2 per cent C.P. NaCl in distilled water. Extractive: 10 cc. physiological saline. Anti-*coli* bacteriophage. Broth pH 7.4.

conditions for preparing concentrated suspensions. Charts 1 and 2 present the results in graphic form, and indicate that a maximum concentration is obtained with a 0.5 per cent agar gel through which the current passes for 18–20 hours.

Failure to detect with ordinary qualitative reactions any traces of protein or protein-derivatives in the purified suspensions led us to determine the total nitrogen content per cc. The following figures are the averages of several analyses of each type of preparation:

(*a*) Non-purified anti-*coli* bacteriophage in Martin's broth: (22 × 10^8 corpuscles/cc.) 0.548 mg. N/cc.

(*b*) Purified anti-*coli* bacteriophage in saline solution: (1 × 10^{16} corpuscles/cc.) 0.092 mg. N/cc.

(*c*) Saline extract of purified agar without phage: 0.048 mg. N/cc.

Consequently, we may attribute to the phage itself (1 × 10^{16} corpuscles) only 0.092 — 0.048 = 0.044 mg. N/cc. This calculates to a maximum of 200 atoms of nitrogen per lytic particle, a number not exceeding the total of nitrogen atoms found in an ordinary protein molecule.

CONCLUSIONS

The method described above, based on the electrophoretic migration of bacteriophage particles into an agar gel and their subsequent re-suspension in a suitable medium, has the following advantages:

It is simple and can be readily carried out on a comparatively large scale by merely inserting additional units between the same electrode cups. It requires but one extraction and the resulting phage suspension is strongly lytic, an average sample being capable of completely lysing susceptible bacteria at a dilution of 10^{-16}. The suspension contains no proteins demonstrable by the biuret, alcohol, xanthoproteic, Millon or Hopkins-Cole reactions and yields but 0.044 mg. N/cc. directly attributable to the phage. Each corpuscle contains no more nitrogen than a single molecule of protein.

In addition the method is applicable to determinations of the electric charge carried by biologically active substances of small dimensions, *e.g.*, phage, toxins, and perhaps some viruses. It offers as well a possible means of purification of these substances.

The purified bacteriophage obtained by such a procedure or similar ones is relatively unstable. Work now in progress indicates that it does not possess nearly the resistance to chemical agents, drying, etc., that non-purified phage displays.

It is suggested that experiments designed to test the therapeutic value of bacteriophage be conducted, when possible, with purified suspensions thereby avoiding any possibility of obscure non-specific reactions due to other constituents of the lysates.

BIBLIOGRAPHY

1. Krueger, A. P., and Tamada, H. T., *Proc. Soc. Exper. Biol. and Med.*, 1929, 26, 530.
2. Bronfenbrenner, J., *Jour. Exp. Med.*, 1927, 14, 873.
Bronfenbrenner, J., Filterable Viruses, Rivers, Williams and Wilkins, 1928, 377.
3. Arnold, L., and Weiss, E., *Jour. Infect. Dis.*, 1925, 37, 411.
4. Todd, C., *Brit. Jour. Exp. Path.*, 1927, 8, 369.
5. Dominikiewicz, M., *Centralbl. f. Bakt., I. Abt., Orig.*, 1908, 47, 5, 666.

MEASUREMENTS OF THE METABOLISM OF TWO PROTOZOANS

By ROBERT EMERSON*

(*From the Laboratory of General Physiology, Harvard University, Cambridge*)

(Accepted for publication, July 21, 1929)

Dr. J. A. Dawson of the Department of Zoology, Harvard University, has been kind enough to place at the writer's disposal relatively large quantities of two protozoans ("*Amoeba proteus*" according to Schaeffer, 1916; and *Blepharisma undulans*) in sufficiently pure condition to make possible some experiments on their metabolism. The results of these experiments, together with some observations on the red pigment of *Blepharisma* are recorded here. Dr. Dawson describes in a separate paper his methods of culturing the organisms.

I

Experiments on Amoeba proteus

The amebae obtained were always accompanied by large numbers of *Chilomonas*. In order to be sure that any metabolism found should refer to *Amoeba proteus* alone, the *Chilomonas* cells, which are much smaller in size, were washed out on a 200-mesh phosphor-bronze sieve, which retained all amebae. After two washings, a microscopical examination of the amebae showed them to be practically free of *Chilomonas*. The former were then concentrated by allowing them to settle in shallow dishes, and decanting. They were washed once or twice on the centrifuge to remove bacteria, and a suspension of them was made up and pipetted into the vessels of Barcroft-Warburg manometers.

Various suspending solutions were tried, similar in composition to Ringer, but much more dilute. None proved successful, however, and metabolism was demonstrable only in distilled water. The water used was commercial distilled water redistilled from a Pyrex still.

Table I gives complete data for one experiment. The temperature was 20°C., and a mixture of 5 per cent CO_2 in air was used. The manometer vessels were the rectangular type, illustrated in a paper by

* National Research Council Fellow.

TABLE I

Respiration of Amoeba proteus

Vessel 6	Vessel 3
$V_F = 7$ cc., 14 mm.³ cells	$v_f = 3$ cc., 6 mm.³ cells
$K_{O_2} = 0.56$	$k_{O_2} = 0.96$
$K_{CO_2} = 1.15$	$k_{CO_2} = 1.22$
$\Delta H = 09.9$ mm. in 130 minutes	$\Delta h = -1.7$ mm. in 130 minutes
$X_{O_2} = -4.5$ mm.³	
$X_{CO_2} = +4.35$ mm.³	
$Q_{O_2} = -1.6$	

the writer (1929), without alkali well. For a detailed explanation of the technique, see Warburg (1926). The notation of Table I is as follows:

V_F = volume of cell suspension.
K_{O_2} = constant of the vessel for oxygen.
K_{CO_2} = constant of the vessel for carbon dioxide.
Δh = pressure change on the manometer.
X_{O_2} = oxygen absorbed.
X_{CO_2} = carbon dioxide evolved.

Capital letters refer to the vessel with the large volume of cell suspension, small letters to the one with the small volume of cell suspension. The vessels contained suspensions of the same density, *i.e.*, the same volume of-cells per cc. X_{O_2} and X_{CO_2} were calculated from these formulae:

$$X_{O_2} = \frac{\Delta H K_{CO_2} - \frac{V_F}{v_f} \Delta h k_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}}$$

$$X_{CO_2} = \frac{\Delta H K_{O_2} - \frac{V_F}{v_f} \Delta h k_{O_2}}{\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}}.$$

X_{O_2} and X_{CO_2} refer to the gas exchange in Vessel number 6, which contained 14 mm.³ cells, over a period of 130 minutes, the time over

which the observation of the values of ΔH and Δh were read. For convenience in comparing with other figures, the value of X_{O_2} is reduced to the oxygen consumption in cubic millimeters per hour per 10 mm.^{3} cells, or Q_{O_2}, equal in this case to -1.6. This figure is much lower than figures published by the writer for green algae, where the value of Q_{O_2} ranged from about -5 to -10. Adolph (1929, p. 313) gives an average value for the oxygen consumption of freshly isolated frog skin, of 133 mm.^{3} O_2 per gm. of fresh weight. Assuming the density of the tissue to be close to 1, frog skin has, according to Adolph's figures, a Q_{O_2} of about -1.33, very close to the writer's figure of -1.6 for *Amoeba*.

Attempts were made to demonstrate anaerobic metabolism, by suspending the cells in media containing glucose or bicarbonate or both, and with an atmosphere of nitrogen which had been passed over red-hot copper to remove oxygen. No anaerobic metabolism was found.

Although it would undoubtedly be of interest to study the characteristics of *Amoeba* respiration, the writer did not undertake further experiments. Owing to the small gas exchange, much larger amounts of cells would be necessary than were available at one time.

II

Experiments with Blepharisma

Blepharisma was available in larger quantities, and consequently was better adapted for these experiments. The cells were free from other protozoans, and bacteria were removed by washing on the centrifuge. Measurements were made with cells suspended in distilled water from a Pyrex still, and in dilute salt solutions. Although the rate of respiration was about the same in either case, it remained constant in salt solution and fell off rapidly in distilled water. Only those experiments made with cells suspended in salt solution are recorded here.

The salt solution was prepared freshly from stock solutions for each experiment. When mixed, its composition was as follows:

Redistilled Water	1 liter
NaCl	500 mg.
KH_2PO_4	25 mg.
$CaCl_2 6H_2O$	100 mg.

In this solution *Blepharisma* cells will live and remain active for several hours without showing any signs of injury. Nothing was found which when added to this solution would cause any substantial increase in respiration. The addition of 1 per cent glucose caused a 10 to 20 per cent increase in oxygen consumption. Peptone in a concentration of 1 mg. per cc. caused a slightly greater increase.

TABLE II

Respiration of Blepharisma

Vessel 6, 87 mm.³ cells	Vessel 3, 37.5 mm.³ cells.
V_F = 7 cc.	v_f = 3 cc.
K_{O_2} = 0.56	k_{O_2} = 0.96
K_{CO_2} = 1.15	k_{CO_2} = 1.22
ΔH = −13 mm. in 25′	Δh = −1.7 mm. in 25 min.
X_{O_2} = −13.1	
X_{CO_2} = +11.7	
Q_{O_2} = −3.6	

Table II records the details of an experiment to determine the oxygen consumption and carbon dioxide production of a sample of *Blepharisma* cells. The notation and method of calculation is the same as for Table I. The temperature was 20°C.

The rate of gas exchange indicated in Table II remained constant for a period of three hours. As in the case of *Amoeba*, the respiratory quotient, $-\frac{X_{O_2}}{X_{CO_2}}$, is nearly equal to unity. The value given for Q_{O_2}, −3.6, is much greater than the figure for *Amoeba*. −3.6 was the lowest value obtained for *Blepharisma*, the average being around −5. With some samples of cells, Q_{O_2} was found to be as high as −7 or −8. These values compare favorably with figures of the writer, for green algae cited above. But they are much smaller than the figures given by Adolph (1929, p. 269) for *Colpoda*. He states that a unit volume of cells uses in an hour four times its volume of oxygen. This means a value of −40 for Q_{O_2}. Adolph says that his figures are more or less in agreement with the values found by other workers for protozoan respiration.

Blepharisma shows a measurable anaerobic metabolism. This is manifest only in the presence of bicarbonate, showing that an acid is evolved under anaerobic conditions, displacing CO_2 from the bicarbonate.

The demonstration of anaerobic metabolism was made as follows:

To 50 cc. salt solution were added 2 cc. of $\frac{M}{10}$ $NaHCO_3$. 80 mm.^{3} of fresh *Blepharisma* cells were washed in this solution and pipetted into Vessel 6. V_F was equal to 7 cc. The gas-space was swept out with nitrogen which had been passed over hot copper to remove oxygen. Nitrogen was passed through for about 5 minutes. This proved long enough to wash out the oxygen dissolved in the cell suspension without removing the CO_2 from the bicarbonate. Controls were made by adding the bicarbonate after saturation with nitrogen.

In the experiment referred to, 80 mm.^{3} cells in an atmosphere of nitrogen at 20°C. showed an increase of pressure of 11 mm. per hour. Assuming this to be due entirely to evolved CO_2, *i. e.*, assuming that nitrogen does not enter into the metabolism, this indicated an evolution of 12.5 mm.^{3} of CO_2 from the bicarbonate per hour. Upon returning the cells to an atmosphere of air, they showed normal respiration. Glucose did not affect the anaerobic metabolism.

Blepharisma cells are colored bright red, and it was thought they might show some special behavior toward light. However, no change in oxygen consumption could be detected when cell suspensions were illuminated by an incandescent lamp or by the light of a Pyrex mercury arc.

SUMMARY

1. The respiration of *Amoeba proteus* was measured. 10 c. mm. of cells were found to use about 1.6 mm.^{3} of oxygen per hour at 20°C. The respiratory quotient was found to be nearly unity.

2. No anaerobic metabolism was found for *Amoeba*.

3. The respiration of *Blepharisma* was found to be from 3 to 7 mm.^{3} oxygen per hour for 10 mm.^{3} cells. The respiratory quotient was about 1.

4. *Blepharisma* was shown to have a definite anaerobic metabolism. 80 mm.^{3} cells caused the evolution of 12.5 mm.^{3} carbon dioxide per hour at 20°C. in the presence of bicarbonate.

CITATIONS

Adolph, E. F., *Jour. Exp. Zoöl.*, 1929, 53, 269, 313.

Emerson, R., Über die Wirkungen von Blausäure, Schwefelwasserstoff, und Kohlenoxyd auf die Atmung Verschiedener Algen, Inaugural Dissertation, Berlin, 1927.

Warburg, O., Über den Stoffwechsel der Tumoren, Julius Springer, Berlin, 1st edition, 1926.

SOME PROPERTIES OF THE PIGMENT OF BLEPHARISMA

By ROBERT EMERSON*

(From the Laboratory of General Physiology, Harvard University, Cambridge)

(Accepted for publication, August 17, 1929)

In connection with measurements of the metabolism of *Blepharisma* described in the preceding paper, the writer made certain observations on the red pigment of this organism. The pigment of the intact organism defies extraction with any of the common organic solvents, but it may easily be obtained in solution by macerating fresh cells in clean quartz sand and extracting with 90 per cent ethyl alcohol.

The alcoholic extract behaves like an indicator. When neutral or acid, it is bright red, the color disappearing in alkali. The turning point is quite near neutrality, and only a slight degree of alkalinity is necessary to make the color-change complete. This may be brought about by adding a drop of M/10 sodium bicarbonate solution to 10 cc. of pigment solution. Perceptible changes in depth of color may be caused by merely varying CO_2 tension.

Fig. 1 shows the absorption curve of the extract of 108 mm.^{3} of cells made up to 10 cc. in ethyl alcohol. The curve was determined on a König-Martens spectrophotometer. ϵ, the extinction coefficient, is plotted against λ, the wave-length in $\mu\mu$. The spectrophotometer readings ϕ_1 and ϕ_2 are shown with their accompanying values of λ and ϵ in Table I.

The curve might be compared with the figure published by E. Ray Lankester in 1873 for the absorption spectrum of the unextracted pigment of *Stentor caeruleus*. Since stentorin is blue and the pigment of *Blepharisma* red, their absorption spectra would not be expected to coincide. But the three maxima shown by the *Blepharisma* pigment in the short-wave-length end of the spectrum might be said to correspond to the three absorption bands of stentorin in the long-wave-length end of the spectrum.

* National Research Council Fellow.

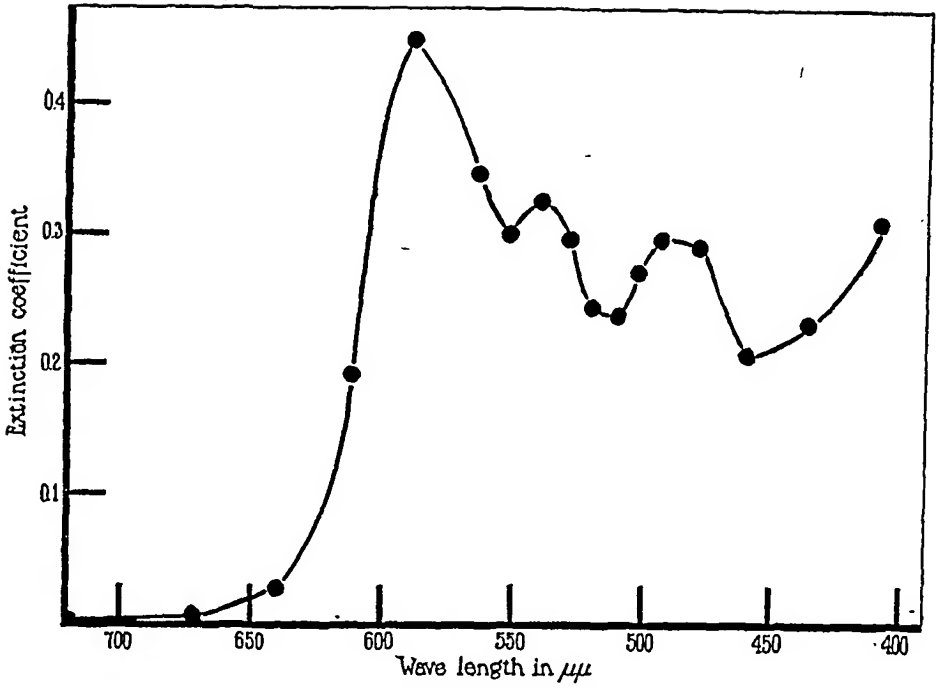


FIG. 1. Curve showing the absorption spectrum of the extracted pigment of *Blepharisma*.

TABLE I

Figures for the Absorption Spectrum of Blepharisma Pigment

λ (μμ)	ϕ_1	ϕ_2	ε
720	42.0	42.0	0
662	41.2	41.2	0
630	42.5	41.0	0.023
602	48.0	36.0	0.186
579	55.7	28.2	0.457
564	53.1	30.6	0.353
552	51.0	31.8	0.300
540	52.0	31.2	0.325
529	51.0	32.0	0.296
520	49.2	33.4	0.245
510	48.9	33.5	0.238
502	49.9	32.5	0.271
494	51.1	32.0	0.297
479	50.7	32.0	0.291
460	Interpolated from another set of cells....		0.208
436			0.232
408			0.308

The pigment fluoresces red in the light of the mercury lamp. This fluorescence is not due to absorption of ultra-violet light, since it appears undiminished when a quinine filter is interposed.

The alcoholic solution of the pigment may be kept for weeks in the refrigerator, but loses its color in a few days at room temperature, even if kept in the dark.

CITATION

Lankester, E. Ray, *Quart. Jour. Micro. Sci.*, 1873, 13, 139.

ON THE BEHAVIOR OF NICKEL CARBONATE IN RELATION TO PHOTOSYNTHESIS

By ROBERT EMERSON*

(From the Laboratory of General Physiology, Harvard University, Cambridge)

(Accepted for publication, July 4, 1929)

Professor E. C. C. Baly (1927, 1928, 1929) claims to have synthesized carbohydrates by the action of visible light on an aqueous suspension of finely divided nickel carbonate saturated with carbon dioxide. He describes the production of weighable amounts of carbohydrate, of the order of 1 gm., by the action of 50 gm. of nickel carbonate.

To produce 1 gm. of glucose from carbon dioxide and water, approximately 0.95 gm. carbon dioxide is necessary. Under standard conditions this amount of carbon dioxide has a volume of 480 cc. One ten thousandth of this amount (48 c. mm.) would be easily measurable in a Barcroft-Warburg manometer, of the type commonly used.

It should therefore be possible to determine whether the appearance of Baly's carbohydrate is accompanied by the disappearance of a corresponding amount of carbon dioxide, and the production of a corresponding amount of oxygen. In any proof that artificial photosynthesis takes place, this should be an important step. It is omitted by Baly and his collaborators.

In this paper the writer describes experiments designed to demonstrate if possible the disappearance of carbon dioxide and the appearance of oxygen, when an aqueous suspension of nickel carbonate is exposed to visible light.

I

Preparation of the Nickel Carbonate

Nickel carbonate was prepared according to Baly's rather meager directions, by the electrolysis of a saturated solution of carbon dioxide in redistilled water with

* National Research Council Fellow.

nickel electrodes.[1] Baly states that with three electrodes 8″ × 5″ × ⅛″ he used 220 volts, and "sufficient resistance intercalated to reduce the current to about 2 amps." He does not state the distance between his electrodes. The writer tried various distances, but was unable to make a similar system to conduct more than 0.5 amps. at 220 volts without any resistance. Using tap water, or commercial distilled water, the system conducted readily. It was concluded that some additional substance must have been present in Baly's system, to give such a high conductivity. If the current density is too low, no nickel carbonate is formed. Traces of various electrolytes were added to redistilled water to make it conduct enough current to produce a good yield of nickel carbonate. Hydrochloric acid was found to be most satisfactory. 0.5 cc. of "C.P." hydrochloric acid diluted 1 to 10 was added to 3 liters of electrolyte.

The precipitated carbonate was centrifuged off, dried to 100°C., heated to 135°–140°C. for 30 minutes, powdered in an agate mortar, passed through a 200-mesh phosphor-bronze sieve, spread in a thin layer in a crystallizing dish covered to protect from dust, and just prior to use illuminated for 12 to 18 hours by a 100 watt lamp at about 7 inches distance.

The substance prepared in this way is surely a basic carbonate of nickel. Heating to 135°–140° turns it a darker green, and it loses CO_2 which it can readily take on again. Heating to higher temperatures blackens it, and all the carbonate is converted to oxide. To obtain some idea of the composition of the preparation, samples which had been dried at 100° and not heated to 135° were weighed into a crucible, ashed, and the loss in weight determined. Assuming the residue to be nickel oxide, the carbonate preparation was found to contain 40 per cent nickel. To determine the amount of CO_2 present in the carbonate, weighed samples were treated with normal sulfuric acid, and the evolved CO_2 measured manometrically. The preparation was found to contain 8.75 per cent CO_2. Gmelin-Kraut (Handbuch der anorgischen Chemie, Heidelberg, 1909, **5**, Part 1, 108–109, 7th edition) states that basic nickel carbonate is of variable composition, and gives as example 3 NiO, CO_2, $6H_2O$. This gives nickel 46.8 per cent, and CO_2 11.7 per cent, figures not differing greatly from those of the writer.

II

Manometric Experiments

Samples of recently irradiated powder were weighed into the bulbs *B* of vessels of the type shown in Fig. 1. The main chamber *C* was given 3 cc. of redistilled

[1] The electrodes were furnished through the courtesy of the International Nickel Company.

water saturated either with 100 per cent CO_2 or with a mixture of 5 per cent CO_2 in air. The vessels were connected with their manometers, filled with the gas used to saturate their contents, and shaken in a thermostat at 20°C. until equilibrium was reached. The contents of the bulb was then washed into the main chamber, without opening the system.

The behavior was followed in continuous light or dark, or in intermittent light, by frequent readings of the manometers. All preparations absorbed carbon dioxide rapidly, absorption being most rapid with 100 per cent CO_2 in air. After mixing, the rate of CO_2 absorption rose rapidly to a maximum, and then fell off slowly, continuing at a

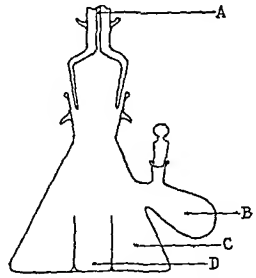


FIG. 1. Type of manometer vessel used. *A*, capillary connecting with manometer; *B*, bulb; *C*, main chamber of vessel; *D*, central well, in which sticks of yellow phosphorus were used.

decreasing rate for 4 or 5 hours. The course of absorption of a typical sample in 100 per cent CO_2 is shown in Fig. 2, total amount of CO_2 absorbed being plotted against time.

The curve shows the absorption of CO_2 by 8.5 mg. of nickel carbonate over a period of 5 hours and 10 minutes. At the end of this time, CO_2 was still being taken up perceptibly, though slowly. In all, 1825 c. mm. of CO_2 were absorbed by 8.5 mg. of carbonate in the period of observation.

The process is reversible at any point. Air may be substituted for 100 per cent CO_2, and the absorbed gas is then evolved. Much of the

CO_2 goes to forming a soluble nickel compound, possibly a bicarbonate, which can be precipitated if the CO_2 is pumped off.

A variety of combinations of CO_2 concentration, light intensity, and nickel carbonate samples were tried, but no effect of illumination could

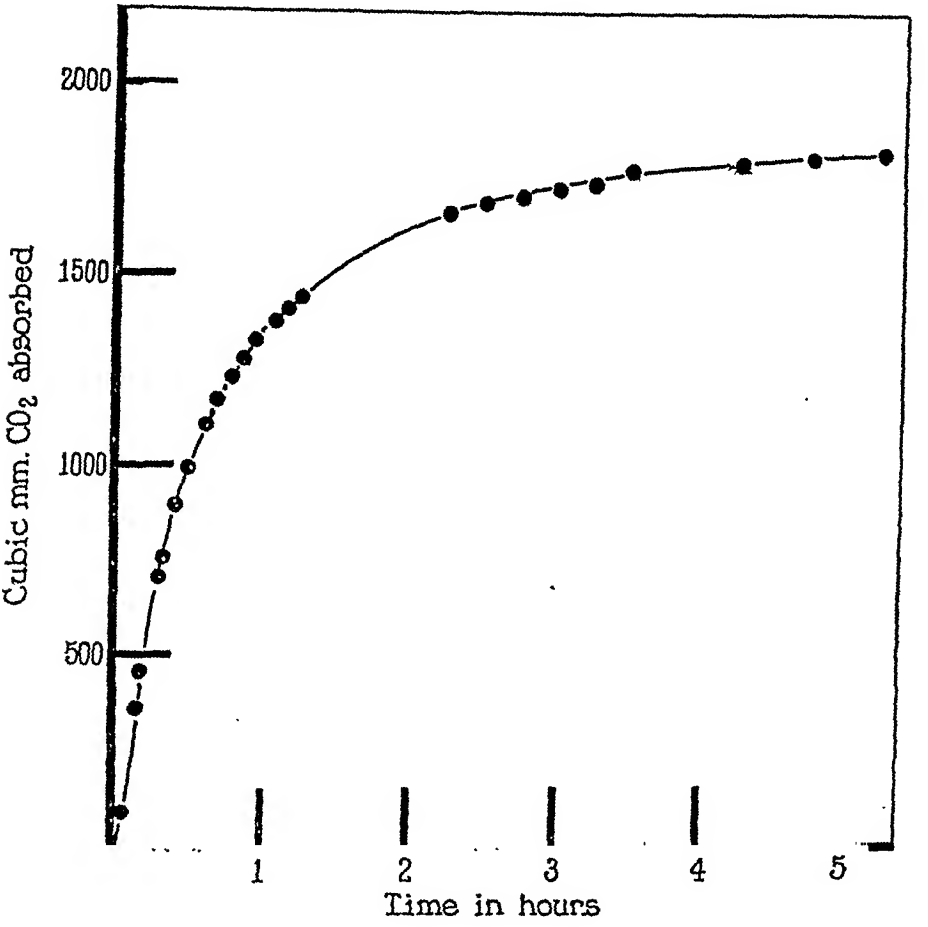


FIG. 2. Curve showing the absorption of CO_2 by 8.5 mg. nickel carbonate. Total CO_2 absorbed is plotted against time.

be observed. Illumination for short and long periods, of intensities from 10,000 to 100,000 Lux were tried, without any effect on the rate of CO_2 absorption.

Baly states that his preparations became inactive through the accumulation of oxygen. Some experiments were therefore tried with

small sticks of white phosphorous in the central well *D* of the manometer vessel, to absorb any possible oxygen, but no difference in behavior could be detected.

III

Gas Analysis Experiments

It was thought possible that a small photosynthesis might be concealed in the rapid absorption of CO_2 which was continuous in light or dark. Several analyses were made of the gas taken from above illuminated samples of nickel carbonate in water, in an attempt to demonstrate the production of oxygen during illumination.

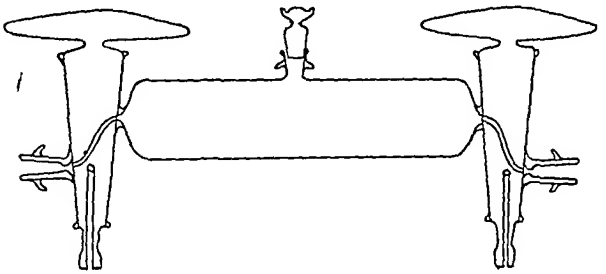


FIG. 3. Type of vessel used for gas-analysis experiments. The long stop-cocks are used to avoid having grease come in contact with the material under investigation.

Vessels of the type shown in Fig. 3, having a total volume of about 12 cc., were filled with 3 or 5 cc. of redistilled water saturated with the desired gas mixture. Just before closing the vessel, 50 to 500 mg. of nickel carbonate were added. The vessel was then shaken in either light or dark in a water thermostat for 2 to 5 hours. Analyses were made with an apparatus of the Haldane type on which one scale division was equal to 10 c. mm. No oxygen was ever detected as a result of illumination.

IV

Direct Test for Carbohydrates

The experiments described above were all carried out in closed vessels, whereas Baly's experiments were made in a continuous stream of carbon dioxide. A system similar to Baly's was devised as follows:

A glass cuvette of about 50 cc. capacity and 9 mm. distance between inside walls was filled with double-distilled water, and a stream of 100 per cent CO_2 was bubbled rapidly through. About 200 mg. of a fresh sample of nickel carbonate was added. The cuvette was illuminated by a 100-watt lamp 25 cm. away for five hours. The nickel carbonate was then centrifuged off, and the supernatant fluid concentrated *in vacuo*. The carbonate which precipitated out during concentration was separated off at intervals. The fluid was concentrated to a fraction of a cubic centimeter, and treated with sulfuric acid. There was no visible charring.

CONCLUSION

The writer was unable to prepare a sample of nickel carbonate which would give any indication of photosynthetic activity. If Professor Baly's preparations are really active, I believe there is some detail in his procedure which he has failed to mention.

LITERATURE CITED

Baly, E. C. C., *Proc. Roy Soc. London, Series A*, 1927, 116, 212.
Baly, E. C. C., *Nature*, October 27, 1928, 122, 651.
Baly, E. C. C., *Proc. Roy. Soc. London, Series A*, 1929, 122, 393.

ELECTROKINETIC PHENOMENA

I. The Adsorption of Serum Proteins by Quartz and Paraffin Oil

By HAROLD A. ABRAMSON

(*From the Laboratory of Research Medicine, Medical Clinic, Johns Hopkins Hospital, Baltimore*)

(Accepted for publication, July 24, 1929)

INTRODUCTION

It is extremely common in laboratory practice to bring a glass, quartz, or paraffin surface into contact with alkaline solutions of dilute serum proteins, but there is not much definite information regarding the adsorption of serum albumin and globulin by these surfaces. The cataphoretic mobility of quartz particles and oil droplets in protein sols will be used here to elucidate more fully the mechanism of adsorption of protein at the phase boundaries between quartz and a paraffin oil with electrolytes. Although the method of cataphoresis does not always show minor variations in quantity or quality of material adsorbed, it nevertheless gives an approach to the determination of any important gross selective adsorption. Quartz and oil droplets seem to adsorb gelatin and egg albumin non-specificially, but this is by no means true for all surfaces. Red cells of different mammals (1), for example, do not change their cataphoretic mobilities in the presence of small amounts of homologous or heterologous serum proteins. The presence of gelatin is equally ineffective. In fact, even in acid solutions of pH 3.6 uninjured red cells still retain their negative charge in the presence of gelatin (unpublished data). This means that the red cell surface is little if at all changed by the presence of relatively great excess of protein molecules.

The biological importance of comparing the cataphoretic mobility of quartz and paraffin oil in the presence of serum is best demonstrated by a few examples. The phagocytosis of inert particles like quartz, car-

bon, manganese dioxide, and so on is frequently studied without sufficient regard to the surface composition of the particles, neglecting the possibility that the particles may adsorb proteins in dilute solution to an extent that would alter the nature of the surface.* A parallel situation exists in studies of adhesiveness of leucocytes to various surfaces. Similarly it is claimed that the prolongation of the clotting time of blood by a paraffin surface is due to a difference between the adsorption of some blood constituent by glass and by paraffin (2).

Davis has studied the effect of serum proteins on the cataphoresis of glass particles (3). At the time of Davis' experiments, the method of micro-cataphoresis had not been so completely developed as it is today.† The differences between the data of this author and those to be reported here are probably due to the difference in method of measurement and in the nature of the adsorbing surfaces. Davis found that in isotonic buffers glass particles adsorb protein from about pH 2.0 to 8.0 (the range investigated), the charge being reversed at pH 4.5 in sols of sufficient concentration where the particles were presumably completely covered with protein.

Methods

Apparatus: The absolute mobility of the particles was determined in a modification of a type of apparatus devised by Northrop and Kunitz (4). An additional change was made in the construction of the non-polarizable electrodes. The agar plug previously used must be changed frequently—as often as once in five days or less. This introduces a serious loss of time. The ideal material would be porous and insoluble.‡ Thus far plaster of paris plugs, although not completely satisfactory, have been very useful. Sufficient powdered gypsum is put into the electrode vessels of the cataphoresis cell and mixed with water *in situ*. The porous plugs are then permitted to harden and dry for several hours. After filling the apparatus with saturated KCl solution, the solution in the apparatus is connected with the plaster plugs, the apparatus is inverted, and the electrode vessels are filled with saturated KCl. About 10 milliamperes are sent through the system for several hours with the electrode vessels still open. This apparently hastens the saturation of the pores of the plaster of paris plug with electrolyte. With a given voltage the current approaches a peak value, indicating that the KCl solution has permeated the entire system. The electrodes are then completed as described previously.

---

*Jones, F. S., *J. Exp. Med.*, 1928, 47, 245.

† Cemented cataphoresis cells were employed.

‡ Insoluble porous cements are being investigated.

The apparatus is not always free from streaming, which is particularly prone to occur with solutions of high conductance.* This streaming can usually be avoided by the following procedure. The apparatus is filled with some solution, preferably saturated KCl, and one stop-cock is turned, completing the connection between atmospheric pressure and the hydrostatic pressure within the electrode. After ten minutes equilibrium is usually reached and the same technic is employed on the other side. After each measurement the pressures are similarly readjusted for a few minutes. These procedures make possible the determination of small differences even though a system of high conductance is involved.

Suspensions: Crude quartz powder of small particle size (Eimer and Amend) was purified by heating with cleaning solution diluted with two parts of water, for about 30 minutes. The particles were allowed to settle and the excess of acid carefully decanted. A large amount of distilled water was added and the quartz was separated by a fine-pored Berkefeld filter. The powder was washed by about 500 cc. of distilled water and then boiled with an excess of fairly concentrated hydrochloric acid and allowed to settle. The filter was again employed to separate and wash the quartz. This washing was continued for several days. Very small particles were obtained by fractional sedimentation. It is important to sterilize the final suspension to prevent the growth of molds.

Emulsions of Nujol (a highly purified paraffin) were easily prepared by shaking equal volumes of Nujol and water or electrolyte very vigorously for about 15 minutes. The larger droplets separated out fairly quickly. Small amounts of this emulsion were added to the protein sols as needed, the volumes being identical in comparative experiments.

The number of particles of quartz and oil per $mm.^3$ in the final suspensions is difficult to determine with accuracy. The suspensions used in the final experiments were delicately cloudy and usually showed about 5–10 particles of 0.5μ to 5μ diameter per field (560 ×) with a blue filter. The same volume of a suspension of quartz particles from a standard suspension was always used.

Serum was obtained from clotted or defibrinated blood. Successive dilutions were made roughly by means of mixing cylinders.

Measurements were made at room temperature (20°–26°C.), in either 0.85 per cent or 0.35 per cent NaCl, the pH being fixed by dilute buffers and measured electrometrically.

EXPERIMENTAL

As is evident from Fig. 1, under the conditions of these experiments surfaces of particles of quartz and paraffin oil are extremely sensitive to

* It can be shown readily that this streaming (current = 0.0045 Amp., specific resistance of electrolyte = 70 Ohms; cross-section of cell = 0.07 $cm.^2$) is most probably produced by some mechanical deficiency in the apparatus rather than to heating by the current.

traces of protein in 0.8 per cent NaCl buffered by M/150 phosphate at pH 7.4.* With less than 1 part of human or rabbit serum in 100,000 parts of solution the mobility is markedly displaced, reaching a level which is fairly constant from 1:10,000 to 1:50. Similar results were obtained with glass particles. Under these conditions, then, the

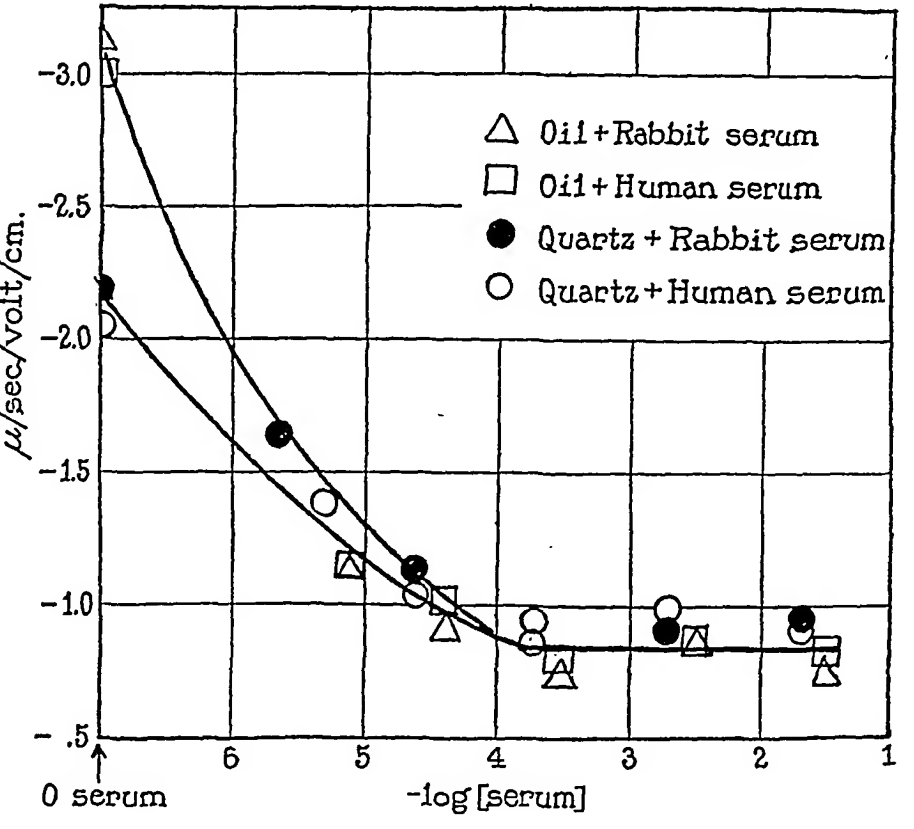


FIG. 1

hydrophilic surface of quartz and the hydrophobic surface of paraffin oil are very similarly attacked by some constituent of the serum, most likely a protein. These curves resemble those obtained with single proteins like gelatin. The data of Davis differ somewhat from those

* The value of about 1.0μ/sec./volt/cm. was obtained for many sera in the low dilutions. One serum gave a value closer to .5μ/sec./volt/cm. The reason for this anomalous behavior is unknown.

just presented. Davis found that in alkaline solutions protein was adsorbed by his glass particles with some difficulty. These differences may be due to differences in material employed or to experimental uncertainties. The experiments here recorded give excellent evidence that under certain circumstances probably a great part of surfaces of paraffin oil or glass are covered even at dilutions of serum of 1:100,000.

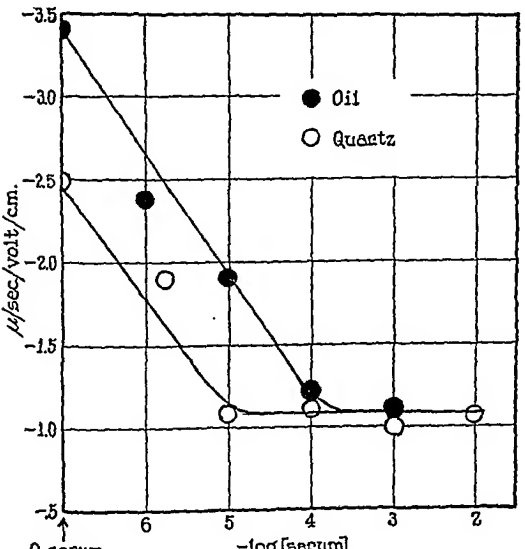


FIG. 2

Similarly, in the presence of leucocytes, red cells, and other systems giving rise to the merest trace of proteins, glass or paraffin oil surfaces must be considered as more or less subject to change, perhaps covered by a film of these proteins.

Fig. 2 shows the same phenomenon occurring at pH 9.3 (borate

buffer) in 0.85 per cent NaCl. Here, as at pH 7.4, the rather high alkalinity does not prevent adsorption of serum protein even in high dilution.

It was of interest to determine whether the parallelism in mobility of quartz and paraffin was observable over a wide range of pH including both sides of the isoelectric points of serum albumin and globulin.

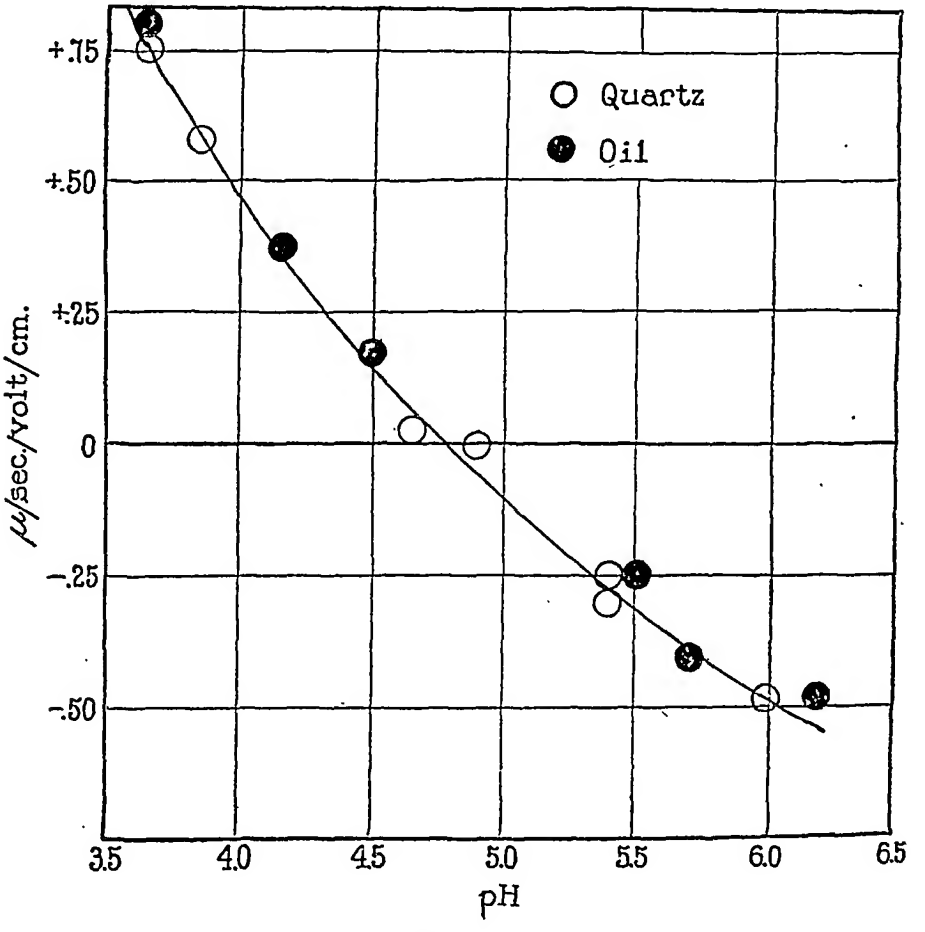


FIG. 3

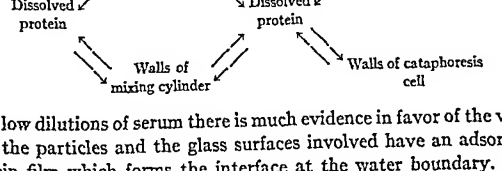
Suspensions of quartz and paraffin oil were studied in 1:50 serum diluted by 0.35 per cent NaCl buffered by N/100 acetate buffers, the ionic strength of the Na-acetate being held constant. In this system quartz and paraffin have fairly identical cataphoretic mobilities

(Fig. 3). Between pH 3.65 and pH 6.0 the curve of mobility as plotted against pH passes through an isoelectric point between 4.7 and 4.8, and resembles the titration curve obtained for serum albumin (5). It is obvious that the presence of electrolytes influences the course of such curves. The theoretical relationship correlating mobility of adsorbed protein and combining power for acids and bases has not yet been described.*

The fact that quartz and droplets of paraffin oil in the presence of serum have an isoelectric point practically identical with that of serum albumin (Davis had noted an isoelectric point of pH 4.5) reminds one of the unsolved problem of the selective adsorption of proteins from solutions containing more than one protein. Here, in the case of serum, the final surface layer seems to consist chiefly of serum albumin, if the experimental isoelectric point be taken as the criterion defining the nature of the adsorbed protein. Whether a globulin surface is first formed and is subsequently covered by albumin must be decided by experiments with purified serum proteins.

DISCUSSION

The equilibrium present in the preceding experiments can be roughly described by the following schema:



In low dilutions of serum there is much evidence in favor of the view that the particles and the glass surfaces involved have an adsorbed protein film which forms the interface at the water boundary. In dilute solutions certain complications are present. For example, in 1:1,000,000 dilution six or more different mixing cylinders are used. In each dilution succeeding the one in which a complete protein film is formed, unknown and varying amounts of protein are adsorbed by

*See Abramson, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 689.

the walls of the mixing cylinders. Thus the *quantity* of dissolved protein present in the final dilution is uncertain. Furthermore, the *nature* of the protein may change. Svedberg and Sjögren (6) have reported that when serum albumin is diluted, decomposition of the albumin molecules occurs. It is evident from the experiments reported here and elsewhere that the mobility (7) imparted by adsorbed protein micells to inert particles at a 1:10,000 dilution is very near that at a 1:100 dilution. What occurs at extremely high dilution is unknown. Another interesting phenomenon observed in this connection in protein sols at high dilutions, *e.g.* at $1:10^7$, with quartz particles in suspension, is the variation in speed amongst the various quartz particles. Some of the particles move as if no protein were present. Others have their velocities lowered slightly—but without the uniformity observable in more concentrated sols. This indicates an uneven distribution of the protein on the particles. When comparatively few protein molecules are available, the equilibrium between particle, vessel wall and dissolved protein is, therefore, most difficult to analyse.*

The practical applications of the preceding remarks in relation to the diluting of sera is apparent. It is advisable at high dilution, 1:1000 or over, to have as little glass surface as possible in contact with the sols. Otherwise the concentration of protein or of immune bodies may turn out to be much lower than initially calculated. Similarly, the difficulties encountered in studies of phagocytosis of inert particles are of interest. Washing the leucocytes and vessels completely free from protein must be difficult, and since the merest trace of adsorbed proteins introduces very complicated systems on the surface of the particles, the comparison of the phagocytosis of dissimilar surfaces such as quartz and paraffin oil in the apparent absence of serum is hardly feasible. If serum is present in sufficient excess so that it can be shown that the particles under investigation are completely and similarly covered by protein films (so that all the particles have the same cataphoretic mobility), the comparison may be more definite (8).

* In low (1:50) serum dilutions anomalous behavior has been rarely observed. For example, a quartz particle during the course of a day's experiments may be observed to be unaffected by the protein although all others in the systems studied were completely covered.

Since cataphoretic velocity is but one function of the particle ion atmosphere, differences in phagocytosis may conceivably still be present even though the cataphoretic mobilities of two different types of particles are the same.

SUMMARY

1. The effect of human and rabbit sera on the cataphoretic mobility of glass and quartz particles, and of paraffin oil droplets, was studied in serum dilutions (with 0.85 per cent NaCl) from 1:50 to 1:1,000,000, over a pH range of 3.6 to 9.3.

2. Under the conditions described, these various types of particles adsorbed protein partially or completely from the most dilute solution giving these particles electrokinetic properties characteristic of certain proteins, probably here those of serum albumin.

3. Quartz particles and paraffin oil droplets both have an isoelectric point between 4.7 and 4.8 in a 1:50 serum dilution.

4. The biological importance of these findings is discussed.

5. A non-polarizable electrode composed of Cu | $CuSO_4$ | $\left.\begin{matrix} CaSO_4 \cdot 2H_2O \\ Sat \cdot KCl \end{matrix}\right|$ is described for use with cataphoresis cells.

I am indebted to Professor L. Michaelis for much valuable advice received in connection with this investigation.

BIBLIOGRAPHY

1. Abramson, H. A., *J. Gen. Phys.*, 1929, 12, 711.
2. Gortner, R. A., and Briggs, D. R., *Proc. Soc. Exp. Biol. Med.*, 1928, 25, 820. Johlin, J. M., *J. Biol. Chem.*, 1929, 81, 99.
3. Davis, H., *Proc. Phys. Soc.*, December 16, 1922, in *J. Phys.*, 1922, 57, p. xvi.
4. Northrop, J. H., and Kunitz, M., *J. Gen. Phys.*, 1925, 7, 729. Abramson, H. A., *J. Gen. Phys.*, 1929, 12, 469.
5. Cohn, E. J., *Physiol. Reviews*, 1925, 5, No. 3, 349.
6. Svedberg, T., and Sjögren, B., *J. Am. Chem. Soc.*, 1928, 50, 3318.
7. Loeb, J., *J. Gen. Phys.*, 1923, 6, 116. Abramson, H. A., Colloid chemistry symposium monograph, VI, Chem. Cat. Co., New York, 1928, 115.
8. Mudd, S., Lucké, B., McCutcheon, M., and Strumia, M., Colloid chemistry symposium monograph, VI, Chem. Cat. Co., New York, 1928, 131.

THE SIZE OF BACTERIA AS THE CAUSE OF THE LOGARITHMIC ORDER OF DEATH

By OTTO RAHN

(From the Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca)

(Accepted for publication, July 5, 1929)

The observation first[1] made by Madsen and Nyman (1907) and independently by Chick (1908) that bacteria killed by almost any cause die in an orderly way which has the same mathematical formulation as the monomolecular reactions, has been verified in a general way by most subsequent investigations. There have been differences of opinion regarding technique; it has been found that the reaction is not always strictly logarithmic, because the "reaction velocity" or the rate of disinfection is not constant but frequently decreases, and this could be accounted for by assuming a varied resistance of the different cells; there has been, in some experiments, the necessity of omitting the first few counts in order to get a reasonable agreement of the death rate. But all experiments made to prove or disprove the claim show that in a general way, the orderly death of bacteria is logarithmic, and that we are justified in speaking of a "logarithmic order of death."

Much less agreement could be obtained on the interpretation of this orderly process. The one extreme of explanation is the assumption that bacteria are small enough to act as molecules, and enter into reaction as any other large molecule would, and therefore must follow the mass law; the mass law reactions are logarithmic. Others scorn

[1] Falk (1923) states that Ikeda (1897) was the first to observe this agreement with the monomolecular law, but it seems to the author that he gives Ikeda too much credit. Ikeda found only that the ratio of times to bring about the same disinfection effect in different concentrations of the same disinfectant is the same. He does not mention any similarity with unimolecular reactions (see also Reichenbach, 1911 and 1922–23).

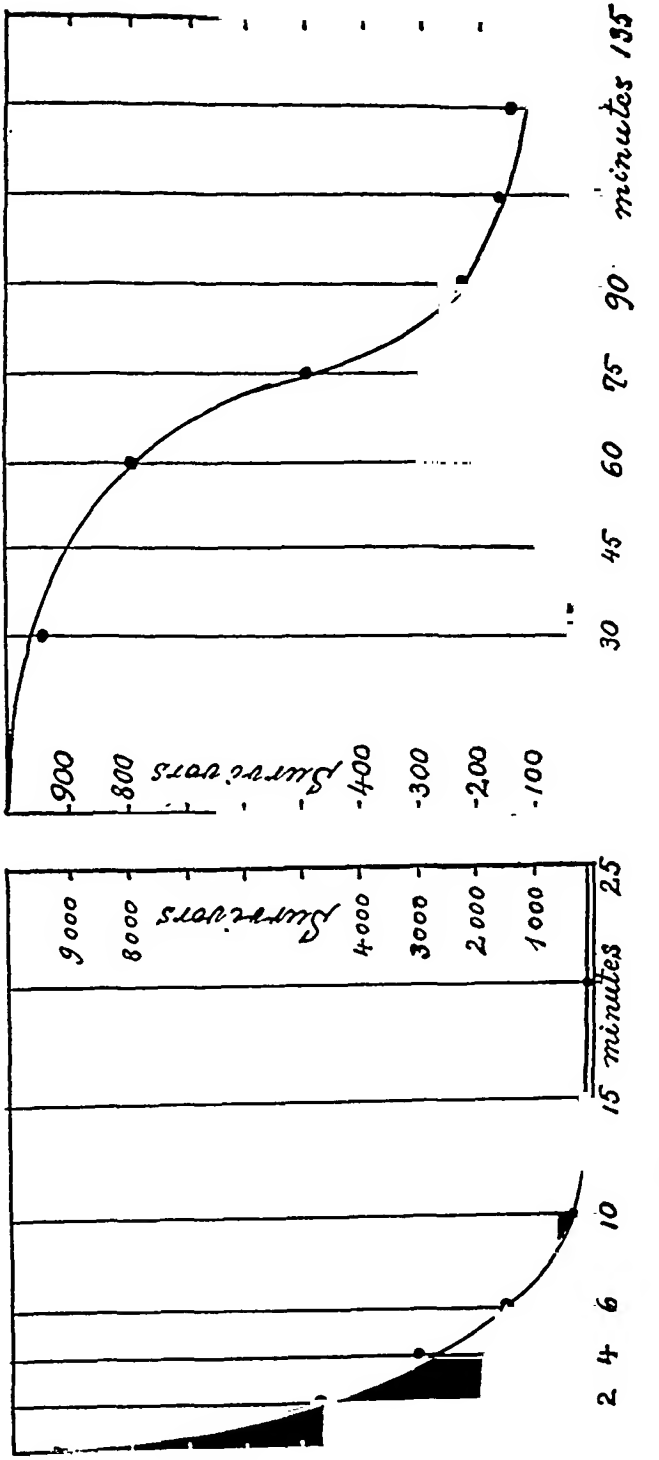


FIG. 1

FIG. 2

FIG. 1. Death of spores of *B. anthracis* by $HgCl_2$. The black line demonstrates the numbers of survivors; the blocks represent the numbers of spores dying per unit time (2 minutes).

FIG. 2. Death of mustard seeds by $HgCl_2$. The black line demonstrates the numbers of survivors; the blocks represent the numbers of seeds dying per unit time (15 minutes).

the idea of comparing such large organisms (with flagella, cell wall, vacuoles and granules indicating a high complexity of composition) with molecules; they believe that the orderly process of death is simply a consequence of an orderly variation of resistance. It is not very easy, however, to fit the laws of chance to the actual facts of the order of death of bacteria, as shall be demonstrated by one of the first experiments on this order, by Madsen and Nyman (1907), and the first experiment for the explanation by variability, by Hewlett (1909).

Fig. 1 shows that the curve of survivors in the case of bacteria spores is distinctly logarithmic, while the survivor curve of the mustard seeds, killed by the same poison and washed with water and treated

TABLE I

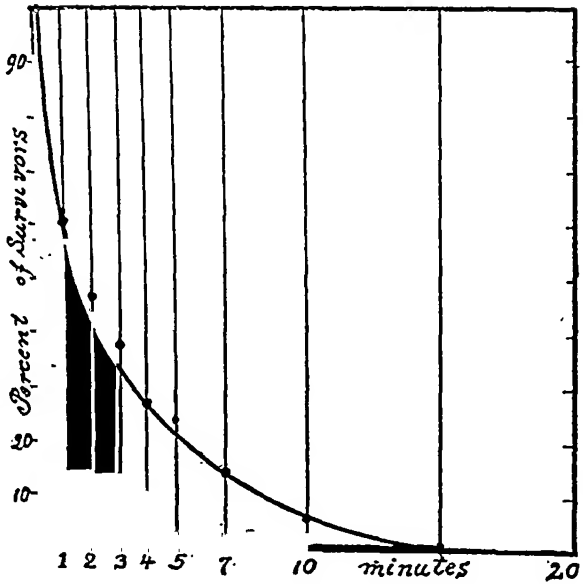
Death Caused by Mercuric Chloride

A Spores of *B. anthracis* in 0.5 per cent $HgCl_2$				B 1,000 Mustard Seeds in 0.2 per cent $HgCl_2$			
Time	Survivors	Spores killed in 2 minutes	$\frac{1}{t}\log\frac{a}{b}$	Time	Survivors	Seeds killed in 15 minutes	$\frac{1}{t}\log\frac{a}{b}$
min.				*min.*			
0	9,500	—	—	30	940.0	(30)	0.0018
2	4,860	4,640	0.146	45	895 8	44.2	0 0016
4	2,964	1,896	0.126	60	790.6	105.2	0.0023
6	1,408	1,556	0 138	75	486.6	304.0	0 0052
10	304	552	0.149	90	220.6	266.0	0 0087
15	2.6	120	0 204	105	163.8	56.8	0 0087
20	1.8	0.3	0.186	120	146.0	17 8	0.0080
25	2.0	0	0 147	135	39.0	107.0	0.0117

with hydrogen sulfide exactly like the bacteria spores, is a typical inverted S-shape. More striking yet is the difference in the "death rate curves" shown by the black blocks and presenting the number of spores or seeds dying in each unit of time. The third difference is the "reaction velocity" or "rate of death" as shown by the K values in Table IA and IB, computed according to the general formula

$$k = \frac{1}{t}\frac{\log a - \log b}{0.434}$$

In Table IA, k is fluctuating but remaining constant within a very large error; in Table IB, however, the death rate is increasing continuously.



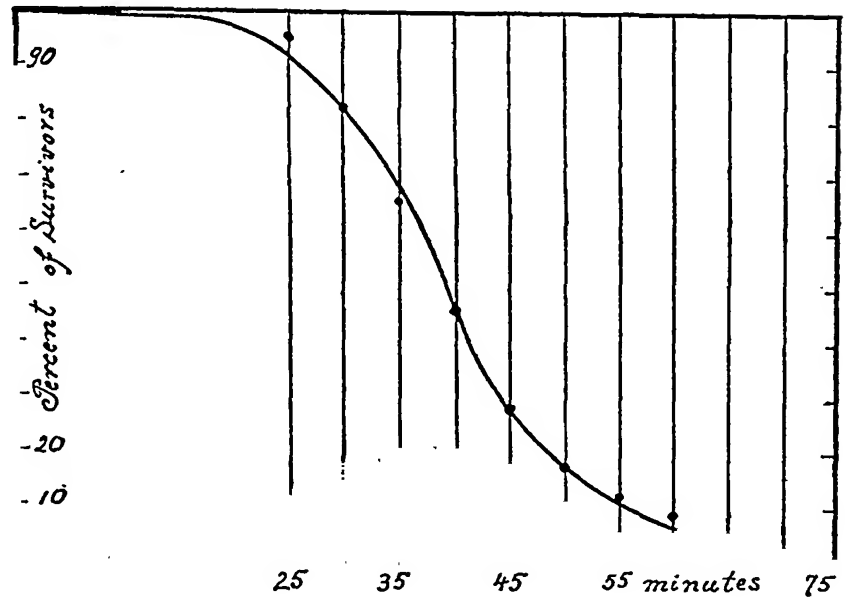


FIG. 3. Death of *Bact. typhosum* by exposure to 49°C.

FIG. 4. Death of *Drosophila* by exposure to 39.45°C.

The black line demonstrates the numbers of survivors; the black blocks represent the percentage of organisms dying per unit time (1 and 5 minutes respectively).

Exactly the same differences between bacteria and multicellular organisms are found if they are exposed to heat, as shown in Table IIA (data by Chick) and B (data by Loeb and Northrop).

If the order of death as actually observed with bacteria is to be explained by variation in resistance, then this variation must be of a special kind. Variation of biological characters generally follows the laws of chance, and the black blocks of Figs. 2 and 4 show the general trend of such curves. Figs. 1 and 3 show how the distribution of resistance would have to be if an approximation to the logarithmic

TABLE II

Death Caused by Heat

A *Bact. typhosum* at 49°C.			*B* Fruit flies, at 39.45°C.		
Time	Survivors	Per cent dying per minute	Time	Per cent of survivors	Per cent dying each 5 minutes
min.			*min.*		
0.28	2,008	—	0	100	—
1.00	1,198	56.0	25	95.5	1.8
2.05	925	13 6	30	83.0	12.5
3	755	18 5	35	65.4	17.6
4	542	10.6	40	45.4	20.0
5	488	2.7	45	28.0	17.4
7	289	4 9	50	17 1	10.9
10	112.8	2 9	55	11.8	5.3
15	24.2	0.9	60	8.8	3.0
20	3.0	0.1	65	2.5	5.3
—	—	—	70	1.1	1.4
—	—	—	75	0.3	0.8

sequence were to be expected. The most sensitive organisms must exist in the largest numbers. That is difficult to explain. Reichenbach (1911) constructed a special theory of growth in order to fit these facts. He assumes that after each doubling of the cells, a certain percentage do not multiply further, but become dormant; these dormant cells are the more resistant the longer they remain in that state. Since even this did not give the correct logarithmic death process, a correction factor was introduced in addition. This assumption seems rather artificial, and does not explain why bacteria spores follow the logarithmic law of death just as accurately or even more so than the vegetative forms.

Neither of these two interpretations of the order of death, which Lee and Gilbert (1918) termed the "mechanistic" and the "vitalistic" theories, has been proved or disproved. No new viewpoint has been added during the last ten years, though the material pro and con has been increased. On account of this deadlock for such a long time, the interest in this question which seems, after all, of considerable biological significance has decreased.

To illustrate how the "mechanists" and "vitalists" each adhere strongly to their theories, a few quotations are given:

Loeb and Northrop (1917): "When we plot the number of flies which die during successive days in terms of percentage of the original number of flies we get that curve of the death rate usually given in life insurance statistics, namely, a probability curve, the ascending branch of which is a little steeper than the descending branch . . . . Miss Chick has stated that bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction, *i.e.* that in each interval of time the same percentage of individuals alive at this time is killed. She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful that the ascending branch became almost a vertical line, thus escaping attention. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time."

Fulmer and Buchanan (1923) (Summary): "It is believed that such resemblances as have been found between such curves (survivor curves plotted against time) and monomolecular reactions or logarithmic curves are superficial and fortuitous. Any method therefore of evaluating disinfecting power based upon such a concept must prove misleading.

"Variations in resistance of individual cells and the distribution of such variations must be regarded as of fundamental importance in accounting for rates of death of organisms."

Lee and Gilbert (1918) (Summary): "It has been proposed to group all these theories (of disinfection) into two classes, namely "vitalistic theories" and "mechanistic theories" . . . . . In view of the experimental evidence which has been presented, disinfection, in the opinion of the authors, must be regarded as an orderly time process which is closely analogous to chemical reaction,—the microorganisms and the disinfectant being regarded as the respective reagents. A definite logarithmic relationship between velocity of disinfection and concentration has been found to exist in all cases investigated. . . . The "theory of graded resistance" as advanced by Eijkman, Hewlett and Reichel has been reviewed, and attention has been directed to the fact that biological characteristics are distrib-

uted as a rule in a manner quite different from that which they have assumed in formulating their theory. In view of these observations, the authors are led to the conclusion that the logarithmic nature of the disinfection process is due to a general *similarity* of the individuals in a given pure culture of microorganisms rather than to a *dissimilarity* of the individuals as postulated in the theories of graded resistances by the supporters of the vitalistic theory."

Cohen (1922): "Subjecting organisms of the colon-typhoid group to mild lethal conditions under moderate temperatures and hydrogen ion concentrations tends to magnify the induction period prior to mortality at the maximum or logarithmic rate. . . . The period of induction is decreased by higher acidity and by higher temperature. It appears to have a duration inversely proportional to some exponent of the temperature. It is analogous to the induction period occurring in chemical reactions. . . . The mortality of bacteria whether by strong disinfectants or by milder agents follows the laws of logarithmic decline. It is shown that the course of the disinfection process can be expressed by mathematical relations comparable to those used in dealing with monomolecular chemical reactions."

A compromise theory similar to that of Lee and Gilbert was attempted by Falk (1923): "Indeed, it is our opinion that the findings of Chick, Brooks, Loeb and Northrop, and of Cohen have all shown that when the material which is being studied is—with respect to the reaction—physiologically homogeneous the course of disinfection, hemolysis, mortality etc. simulates the well-known logarithmic curve of mass action chemical processes. When the viable material is lacking in homogeneity, *i.e.* as between young and old cells, or as between vegetative and spore forms in Dr. Chick's work, the curve which describes the course of the reaction varies from the logarithmic."

This theory seems to be given up later by Falk in favor of an explanation by multimolecular reaction as it appears from the following quotation:

Falk and Winslow (1926): "Where disinfection does not follow a logarithmic course, and is not to be described by the unimolecular equation, the course of the process may sometimes be described by the equation of a bi-, tri-, or higher multimolecular reaction. Such an explanation may render unnecessary the assumption of variability in biological resistance to account for deviation from a logarithmic mortality curve."

Whenever two groups of thorough research workers hold such opposed views as in this case, it is fairly safe to assume that some essentially new principle is involved which none of the two parties realized.

A comprise seems difficult, but it is so largely because the "mechanists" do not pay sufficient attention to the deviations from the strict logarithmic law, while the "vitalists" refuse largely to acknowledge the facts, and base their refusal upon an analogy with multicellular organisms which may not be altogether correct, as shall be proved

presently. One more reason for considerable confusion in disinfection experiments is the difficulty in obtaining identical death rates with subcultures of the same strain on different days. The death rates vary so enormously that an unknown factor, perhaps of catalytic nature, must be assumed. But this has really nothing to do with the order of death; it affects the rate only, and the present discussion is altogether limited to the order of death.

The attempt to explain the logarithmic order of death as a consequence of the small size of bacteria shall be limited here to the death by heat. It is based upon the assumption that death is caused by some chemical change taking place in the cell, and that this chemical process as such follows the same laws as all other chemical processes. Possibly, this reaction is a coagulation of a certain cell protein, or it is the inactivation of an enzyme essential for life, or of some other thermolabile cell compound. The heat coagulation of proteins follows the mass law, and is a monomolecular process (Chick and Martin, 1910); so is the heat destruction of enzymes (Tammann, 1895). It would be not at all surprising that the chemical process causing death by heat is monomolecular. This process, whatever it be, is called by Brooks (1918) the "fundamental reaction."

Thus far, probably, the vitalists will agree. But they will say at once that as long as all cells are alike, the same reaction must proceed in exactly the same way in all cells, and if 20 per cent of the protoplasm in one cell is coagulated, there must be 20 per cent coagulated in everyone of the cells. They must all die at exactly the same moment if they are alike, and the fact that they do not all die simultaneously is the best proof for variation in resistance. This argument can be met by introducing size as a factor.

Let us consider this process of heat coagulation in more detail. If we place a beaker with an albumin solution in a water bath of 65°C., the mass of protein will be gradually coagulated according to the mass law. If this solution were divided by a partition into two independent halves, it will be generally agreed upon that, upon heating in the water bath, the processes will be exactly parallel in the two liquids. If we divide the mass of protoplasm evenly into 1000 separate units, the process will be parallel in each of the units, and when half of the protein is coagulated in one of them, we are certain that exactly the same has happened in each of the other 999.

And yet, this parallelism is not unlimited, because finally, if we continue to divide the total mass of protein, there will be only one molecule left to each division. What will happen when this liquid divided up into these smallest units, each containing only one molecule of protein, is placed into the water bath of 65°C.? The molecules will react exactly in the same way as if they were in a continuous liquid, as if there were no partitions; they will follow the mass law. The division into smallest units can make no alteration in the process, because the protein molecules do not react with each other, at least not primarily. They either react with water in which they are suspended, or they give off water; neither of these processes could be altered by partitions in the medium.

This means, then, that the sum of all these units would have to follow the logarithmic law of mass action. In this case where each unit contains only one reacting molecule, some molecules will react faster then others, and consequently, some units will contain changed molecules and some will not, even though they were exactly alike at the start, and had been treated in exactly the same way. This division into the smallest possible units makes the mass law a fallacy. The mass law holds true only where the number of reacting molecules is practically innumerable. If the number of molecules is limited, the laws of chance enter. It will be shown later than even with 100 reacting molecules per cell, the chance is not altogether excluded.

The extreme theory that the entire protoplasm is one giant molecule need not be assumed for our purpose here. Doubtless the smallest bacteria cell contains many protein molecules though they may be interlinked somehow. These molecules are not all alike. There must be some specialization corresponding to that of the chromosomes in plant cells. Each section of each chromosome is different as the geneticists have shown. It is not a very bold theory to assume that one of these sections is essential for growth or multiplication, and with its destruction, the cell loses the power of multiplication and is considered dead according to the plate count technique of the bacteriologist.

This same order would hold true if there were several such essential molecules in each cell, and the destruction of any one of them would cause death. If, however, two molecules of this most essential type

must be destroyed before multiplication ceases, then another law enters. On the following pages, an equation for the order of death is developed for any number of reacting molecules.

If a bacteria per unit space (*e.g.* per cc.) are exposed to some definite unfavorable temperature which will inactivate m molecules of protoplasm in m cells per unit time, the rate of inactivation of the molecules is $\frac{m}{a}$. The portion not acted upon is $1 - \frac{m}{a}$.

If the destruction of *one* molecule per cell would cause the bacterium to die the numbers of survivors and of dead bacteria would be

After the time	Survivors	Dead cells
0 units	a	0
1	$a\left(1 - \frac{m}{a}\right)$	m
2	$a\left(1 - \frac{m}{a}\right)^2$	$m\left(1 + 1 - \frac{m}{a}\right)$
3	$a\left(1 - \frac{m}{a}\right)^3$	$m\left[1 + \left(1 - \frac{m}{a}\right) + \left(1 - \frac{m}{a}\right)^2\right]$

We substitute for the expression $\left(1 - \frac{m}{a}\right)$ which returns again and again, the letter q, and obtain for the time n the expressions

n	$a\,q^n$	$m(1 + q + q^2 + q^3 + \ldots + q^{n-1})$
		$= m\left(\frac{1 - q^n}{1 - q}\right) = a(1 - q^n)$

The decrease of the survivors is logarithmic; $q = 1 - \frac{m}{a}$ is the proportion of cells not acted upon in unit time.

The next case would be the assumption that the cell can recover if only one molecule is destroyed, but will die if two molecules are inactivated. We proceed as above:

Time	Uninjured	Injured	Dead
0	a	0	0
1	aq	m	0

Time	Uninjured	Injured	Dead
2	aq^2	$2mq$	$\frac{m^2}{a}$
3	aq^3	$3mq^2$	$\frac{m^2}{a}(1 + 2q)$
n	aq^n	nmq^{n-1}	$\frac{m^2}{a}\left[1 + 2q + 3q^2 + \ldots + (n-1)\,q^{n-2}\right]$. $= a[1 + nq^{n-1} + (n-1)\,q^n]$

If we assume that 3 molecules have to be destroyed before the cell cannot recover, we will have four groups of cells:

Time	Uninjured	⅓ Injured	⅔ Injured	Dead
o	a	0	0	0
1	aq	m	0	0
2	aq^2	$2mq$	$\frac{m^2}{a}$	0
3	aq^3	$3mq^2$	$\frac{3m^2}{a}q$	$\frac{m^2}{a^2}$
4	aq^4	$4mq^3$	$\frac{6m^2}{a}q^2$	$\frac{m^2}{a^2}(1 + 3q)$
n	aq^n	nmq^{n-1}	$\frac{n(n-1)}{2}\frac{m^2}{a}q^{n-2}$	$\frac{m^2}{a^2}\left[1 + 3q + 6q^2 + \ldots. \ldots + \frac{(n-1)(n-2)}{2}q^{n-3}\right]$

It is desirable to get a general formula for any number of molecules r necessary to be destroyed to insure the death of the cell. Thanks to the experience and kind assistance of Dr. W. A. Hurwitz, of the Department of Mathematics, Cornell University, such a general formula could be derived by studying the development of the formulae so far mentioned. If we chose, *e.g.* the number of dead cells after the

time n which we may call Dn, we find the following regularity in the development:

1 molecule: $m[1 + q + q^2 + q^3 + \ldots\ldots + q^{n-2} + q^{n-1}] = Dn^{(1)}$

2 molecules: $\frac{m^2}{a}\left[1 + 2q + 3q^2 + \ldots\ldots\ldots (n-1)\, q^{n-2}\right] = Dn^{(2)}$

3 molecules: $\frac{m^3}{a^2}\left[1 + 3q + 6q^2 + \ldots\ldots \frac{(n-1)\,(n-2)}{2}\, q^{n-3}\right] = Dn^{(3)}$

The regularity of development consists in this: the expression in the second equation after $\frac{m^2}{a}$, is the first derivative of the corresponding expression in the preceding line, *i.e.* the factor of m; in the same way, with 3 molecules, the sum $[1 + 3q + \ldots]$ is one-half of the derivative of the preceding expression, $[1 + 2q + \ldots]$. If we continue this systematic development, and call the total sum of the expression for 1 reacting molecule $= f(q)$, we find:

$$Dn^{(1)} = mf(q)$$

$$Dn^{(2)} = \frac{m^2}{a} f'(q)$$

$$Dn^{(3)} = \frac{m^3}{2\,a^2} f''(q)$$

$$Dn^{(4)} = \frac{m^4}{a^3\,(3)!} f'''(q)$$

$$Dn^{(r)} = \frac{m^r}{a^{r-1}} \frac{1}{(r-1)!} f^{(r-1)}(q)$$

The original $f(q)$ was found to be $1 + q + q^2 + q^3 + \ldots + q^{n-1}$. Summarized, this gives

$$f(q) = \frac{1 - q^n}{1 - q}$$

Thus, for the case that inactivation of only one molecule is sufficient to cause death, we have the equation

$$(1 - q)\, f(q) = 1 - q^n$$

This equation, differentiated, gives

$$(1 - q) f'(q) - f(q) = - nq^{n-1}$$

If this is differentiated again, we obtain

$$(1 - q) f''(q) - f'(q) - f'(q) = - n(n - 1) q^{n-2}$$

$$(1 - q) f''(q) - 2f'(q) = - n(n - 1) q^{n-2}$$

The next differentiation will give us the expression for the case where 4 molecules must be destroyed to cause death:

$$(1 - q) f'''(q) - 3f''(q) = - n(n - 1) (n - 2) q^{n-3}$$

The order of progression is plain, and we can write the reaction equation for r molecules:

$$(1 - q) f^{(r-1)}(q) - (r - 1)! f^{(r-2)}(q) = - n(n - 1) (n - 2) \ldots (n - r + 2) q^{n-r+1}$$

This equation is multiplied with $\frac{m^r}{a^{r-1} (r - 1)!}$

$$\frac{m(1 - q) f^{(r-1)}(q)}{a^{r-1} (r - 1)!} - \frac{f^{(r-2)}(q) m^r}{(r - 2)! a^{r-1}} = \frac{- n(n - 1) (n - 2) \ldots (n - r + 2)}{(r - 1)!} q^{n-r+1} \frac{m^r}{a^{r-1}}$$

We now substitute in this equation the value for $Dn^{(r)}$ from the preceding page, and also the value for $Dn^{(r-1)}$:

$$Dn^{(r)} (1 - q) - \frac{m}{a} Dn^{(r-1)} = \frac{- n(n - 1) (n - 2) \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^r}{a^{r-1}} \cdot q^{n-r+1}$$

Since $1 - q = \frac{m}{a}$, we get

$$Dn^{(r)} = Dn^{(r-1)} - \frac{n(n - 1) (n - 2) \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^{r-1}}{a^{r-1}} \cdot q^{n-r+1}$$

Thus, for any r-value, the number of dead cells after the time n is given by the number of the dead cells for the reaction of the next lower order; if we just continue this, we must finally come to $r = 1$

time n which we may call Dn, we find the following regularity in the development:

1 molecule: $m[1 + q + q^2 + q^3 + \ldots\ldots + q^{n-2} + q^{n-1}] = Dn^{(1)}$

2 molecules: $\frac{m^2}{a}\left[1 + 2q + 3q^2 + \ldots\ldots\ldots (n-1)\, q^{n-2}\right] = Dn^{(2)}$

3 molecules: $\frac{m^3}{a^2}\left[1 + 3q + 6q^2 + \ldots\ldots \frac{(n-1)\,(n-2)}{2}\, q^{n-3}\right] = Dn^{(3)}$

The regularity of development consists in this: the expression in the second equation after $\frac{m^2}{a}$, is the first derivative of the corresponding expression in the preceding line, *i.e.* the factor of m; in the same way, with 3 molecules, the sum $[1 + 3q + \ldots]$ is one-half of the derivative of the preceding expression, $[1 + 2q + \ldots]$. If we continue this systematic development, and call the total sum of the expression for 1 reacting molecule $= f(q)$, we find:

$$Dn^{(1)} = mf(q)$$

$$Dn^{(2)} = \frac{m^2}{a} f'(q)$$

$$Dn^{(3)} = \frac{m^3}{2\,a^2} f''(q)$$

$$Dn^{(4)} = \frac{m^4}{a^3\,(3)!} f'''(q)$$

$$Dn^{(r)} = \frac{m^r}{a^{r-1}} \frac{1}{(r-1)!} f^{(r-1)}(q)$$

The original $f(q)$ was found to be $1 + q + q^2 + q^3 + \ldots + q^{n-1}$. Summarized, this gives

$$f(q) = \frac{1 - q^n}{1 - q}$$

Thus, for the case that inactivation of only one molecule is sufficient to cause death, we have the equation

$$(1 - q)\, f(q) = 1 - q^n$$

This equation, differentiated, gives

$$(1 - q)\, f'(q) - f(q) = - nq^{n-1}$$

If this is differentiated again, we obtain

$$(1 - q)\, f''(q) - f'(q) - f'(q) = - n(n - 1)\, q^{n-2}$$

$$(1 - q)\, f''(q) - 2f'(q) = - n(n - 1)\, q^{n-2}$$

The next differentiation will give us the expression for the case where 4 molecules must be destroyed to cause death:

$$(1 - q)\, f'''(q) - 3f''(q) = - n(n - 1)\, (n - 2)\, q^{n-3}$$

The order of progression is plain, and we can write the reaction equation for r molecules:

$$(1 - q)\, f^{(r-1)}(q) - (r - 1)!\, f^{(r-2)}(q) = - n(n - 1)\, (n - 2) \ldots (n - r + 2)\, q^{n-r+1}$$

This equation is multiplied with $\dfrac{m^r}{a^{r-1}\,(r - 1)!}$

$$\frac{m(1 - q)\, f^{(r-1)}(q)}{a^{r-1}\,(r - 1)!} - \frac{f^{(r-2)}(q)\, m^r}{(r - 2)!\, a^{r-1}} = \frac{- n(n - 1)\, (n - 2) \ldots (n - r + 2)}{(r - 1)!}\, q^{n-r+1}\, \frac{m^r}{a^{r-1}}$$

We now substitute in this equation the value for $Dn^{(r)}$ from the preceding page, and also the value for $Dn^{(r-1)}$:

$$Dn^{(r)}\,(1 - q) - \frac{m}{a} Dn^{(r-1)} = \frac{- n(n - 1)\, (n - 2) \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^r}{a^{r-1}} \cdot q^{n-r+1}$$

Since $1 - q = \dfrac{m}{a}$, we get

$$Dn^{(r)} = Dn^{(r-1)} - \frac{n(n - 1)\, (n - 2)\, \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^{r-1}}{a^{r-1}} \cdot q^{n-r+1}$$

Thus, for any r-value, the number of dead cells after the time n is given by the number of the dead cells for the reaction of the next lower order; if we just continue this, we must finally come to $r = 1$

and this case is known. The number of dead cells can therefore be computed for any value of r and n. Substituting the letter C_r for the long expression to be substracted from $Dn^{(r-1)}$, we obtain

$$Dn^{(r)} = Dn^{(r-1)} - C_r$$

$$Dn^{(r-1)} = Dn^{(r-2)} - C_{r-1}$$

$$Dn^{(r-2)} = Dn^{(r-3)} - C_{r-2}$$

$$\vdots$$

$$Dn^{(2)} = Dn^{(1)} - nmq^{n-1}$$

The number of dead cells is then really the total sum of all the C_r-values substracted from the last member $Dn^{(1)}$, which is known to equal $a\ (1 - q^n)$ (p. 188). This gives the following value for $Dn^{(r)}$:

$$Dn^{(r)} = a(1-q^n) - nmq^{n-1} - \frac{n(n-1)}{2}\frac{m^2}{a}q^{n-2} - \ldots. - \frac{n(n-1)\ldots(n-r+2)}{(r-1)!}\cdot\frac{m^{r-1}}{a^{r-2}}\cdot q^{n-r+1}$$

We substitute

$$1 - q = p = \frac{m}{a}$$

$$Dn^{(r)} = a\left(1 - q^n - nq^{n-1}p - \frac{n(n-1)}{2}q^{n-2}p^2 - \ldots\ldots - \frac{n(n-1)\ldots(n-r+2)}{(r-1)!}\cdot q^{n-r+1}\cdot p^{r-1}\right)$$

This is the final expression for the total number of dead cells after the time n, when r molecules per cell must be destroyed before the cell is dead.

This final equation does not mean very much to the average biologist (including the author) who is not trained along statistical lines, and the only way to make it intelligible is to apply it to some simple case.

Unfortunately, it has not as yet been possible to condense this long expression to a simpler form, and the application means therefore a very tedious computation of many data. By choosing very simple conditions, however, the amount of work can be reduced a little.

The following example is calculated on the assumption that 1,000,000 cells are acted upon by some harmful influence at such a rate that 90 per cent of the protoplasm molecules are inactivated per unit time. In the terms of our equation, this means

$$a = 1{,}000{,}000$$

$$p = \frac{m}{a} = 0.9$$

$$q = 1 - \frac{m}{a} = 0.1$$

n is the unit of time. In Table III, the number of survivors is calculated for the end of each unit of time until there is less than one living cell left. This calculation is carried through for successive r-values from 1 to 12; that means, for the assumption that the destruction of 1, 2, 3, etc. molecules means the death of the cell. The table gives the total number of survivors, and the cells dying per unit time.

The cells dying per unit time are also plotted in the block curves (Fig. 5) which will show to most readers more distinctly than the figures how the curve gradually changes its shape. For $r = 1$, it is plainly logarithmic; for $r = 2$, it appears practically of the same shape, except that the first time unit shows no deaths. Even for $r = 6$, the general shape of the curve resembles the first one except that it is flattened. For a while, the largest number of deaths occurs in the first unit of time in which any death takes place at all. However, at $r = 9$, the number of deaths in the first and second dying period are equal, and for $r > 9$, more organisms die in the second than in the first time unit. That this change occurs at $r = 9$, is, of course, the result of our choice of $q = 0.9$; but regardless of how we chose q, there will always be a r-limit beyond which the death in the first time unit is smaller than in the second. The maximum of the death rate shifts to third place for $r = 2 \times 9$, and to fourth place for $r = 3 \times 9$.

TABLE III

A Theoretical Case of Disinfection

Time n	$r = 1$	$r = 2$	$r = 3$	$r = 4$	$r = 5$	$r = 6$	$r = 7$	$r = 8$	$r = 9$	$r = 10$	$r = 11$	$r = 12$
	Survivors											
0	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000 000
1	100,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000 000
2	10,000	190,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
3	1,000	28,000	271,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
4	100	3,700	52,300	343,900	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
5	10	460	8,560	81,460	409,510	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
6	1	55	1,270	15,850	114,265	468,559	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
7	0	6	176	2,728	25,691	149,693	521,703	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
8	0	1	23	432	5,024	38,092	186,894	569,533	1,000,000	1,000,000	1,000,000	1,000,000
9	0	0	3	64	891	9,331	52,972	225,159	612,580	1,000,000	1,000,000	1,000,000
10	0	0	0	8	147	1,634	12,794	70,190	225,159	651,322	1,000,000	1,000,000
11	0	0	0	1	23	396	2,749	18,533	52,972	263,901	686,189	1,000,000
12	0	0	0	0	4	50	541	4,329	8,331	70,191	302,643	717,570
13	0	0	0	0	1	7	99	920	891	12,795	89,562	340,997
14	0	0	0	0	0	1	13	180	64	1,635	18,535	110,870
15	0	0	0	0	0	0	3	34	3	147	2,751	25,637
16							0	6	0	9	295	4,330
17							0	1	0	0	24	541
18							0	0	0	0	2	50
19							0	0	0	0	0	3
20							0	0	0	0	0	0

Cells Dying per Unit Time

1	900,000	0	0	0	0	0	0	0	0	0	0	0
2	90,000	810,000	0	0	0	0	0	0	0	0	0	0
3	9,000	162,000	729,000	0	0	0	0	0	0	0	0	0
4	900	24,300	218,700	656,100	0	0	0	0	0	0	0	0
5	90	3,240	43,740	262,440	590,490	0	0	0	0	0	0	0
6	9	405	7,290	65,610	295,245	531,441	0	0	0	0	0	0
7	—	59	1,094	13,124	88,566	318,866	478,297	0	0	0	0	0
8	—	7	153	2,294	20,665	111,600	334,808	430,467	0	0	0	0
9	—	1	20	367	4,133	29,760	133,923	344,373	387,421	0	0	0
10	—	—	3	55	744	6,697	40,178	154,969	387,421	348,679	0	0
11	—	—	—	8	124	1,338	10,046	51,658	172,187	387,421	313,811	0
12	—	—	—	1	20	245	2,908	14,204	44,641	193,710	383,546	282,430
13	—	—	—	—	3	42	441	3,408	7,440	57,396	213,081	376,573
14	—	—	—	—	—	7	82	740	826	11,160	71,027	230,128
15	—	—	—	—	—	1	4	148	61	1,488	15,784	85,232
16							3	28	3	138	2,455	21,308
17							—	5	—	8	272	3,788
18							—	—	—	—	22	491
19							—	—	—	—	1	47
20							—	—	—	—	—	3

With increasing r, this block curve becomes more and more symmetrical.

To prove this point, the survivors for $r = 100$ molecules were computed. These figures show a type of curve absolutely different from

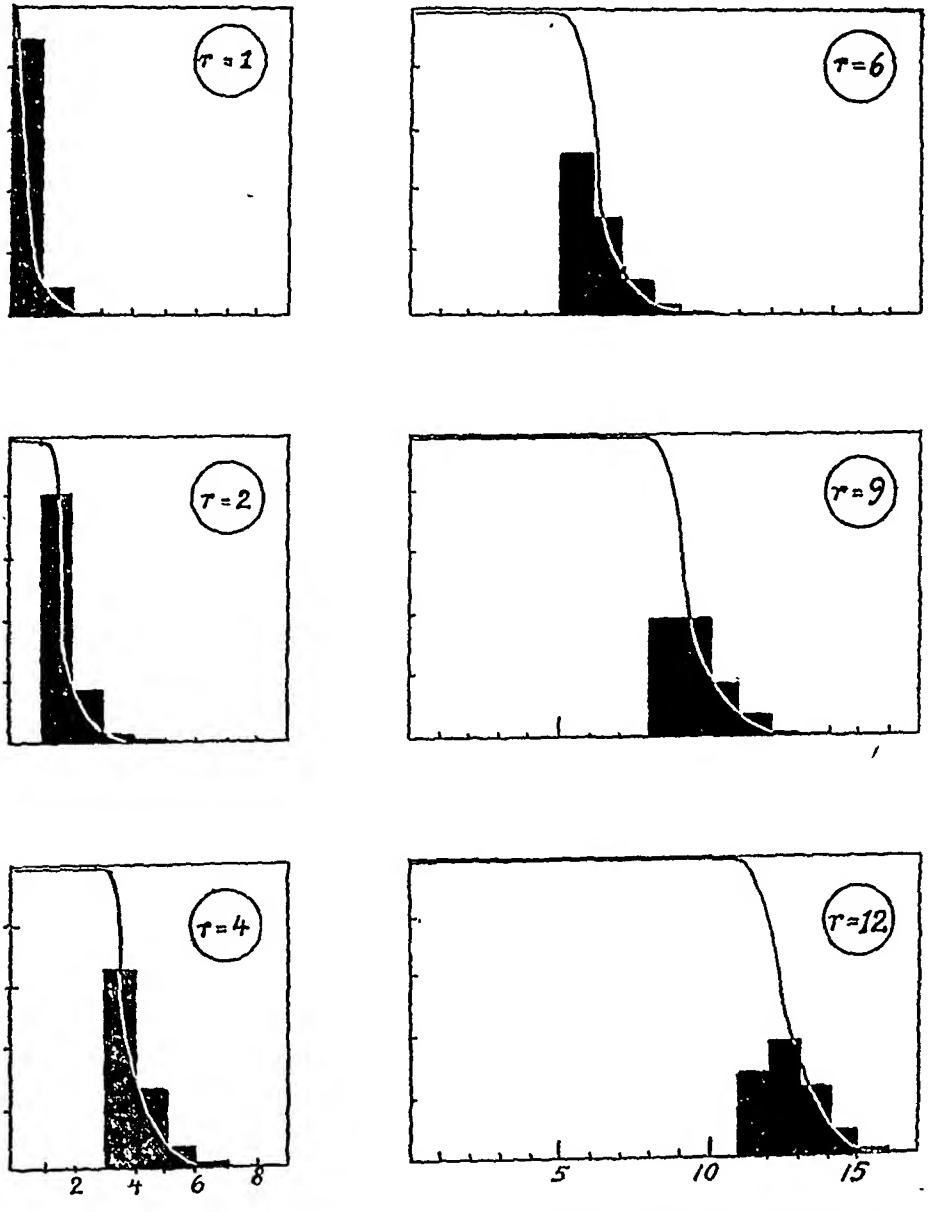


FIG. 5. Survivor curves and deaths per unit time for different numbers of reacting molecules per cell

that for $r = 1$; it resembles, to the author's unprejudiced mind, a variability curve, and with this curve before one's eyes, it is difficult to realize that it represents the order of death of absolutely uniform organisms which have all exactly the same resistance. This order of death is brought about by the circumstance that with 100 molecules, the reaction is not yet uniform in all cells, and it is the law of chance that still rules.

Just as conspicuous as the gradual change of shape of the black curves of deaths per unit time is the fact that for any r larger than 1,

TABLE IV

The Case of 100 Reacting Molecules in the "Theoretical Case of Disinfection"

Time	Survivors	Dying per unit time	Time	Survivors	Dying per unit time
1–99	1,000,000	0	115	39,827	32,629
100	999,973	27	116	20,567	19,260
101	999,678	295	117	9,993	10,574
102	998,055	1,623	118	4,575	5,418
103	992,163	4,892	119	1,977	2,598
104	976,288	15,875	120	807	1,170
105	942,422	33,866	121	312	495
106	882,643	59,779	122	114	198
107	793,748	88,895	123	40	74
108	678,925	114,823	124	14	26
109	548,509	130,416	125	5	9
110	416,644	131,865	126	2	3
111	296,767	119,877	127	1	1
112	197,979	98,788			
113	123,677	74,302			
114	72,456	51,221			

there is no death at all for a certain time; this time increases in direct ratio with r. We must expect this; if a large number of molecules must be destroyed before the cell has lost the power of recovery, it is very improbable that all the molecules in one cell will be destroyed in the first time unit. This improbability increases with the number of reacting molecules. If, however, only one molecule per cell exists, the largest number of deaths in the first unit of time is unavoidable.

The dominance of the logarithmic order of death with bacteria seems to indicate that they contain, among others, one peculiar molecule

extremely sensitive to heat whose inactivation prevents any further multiplication; or there may be several such molecules, but the inactivation of any one of them means death. In many instances, however, the death rate $\frac{1}{t} ln \frac{a}{b}$ is not constant, but decreases. In a few cases it increases. It becomes necessary to study the meaning of this, and to compute the death rates for the theoretical cases present in Table III.

The results in Table V show that the death rate computed as in ordinary disinfection experiments is distinctly increasing in all cases

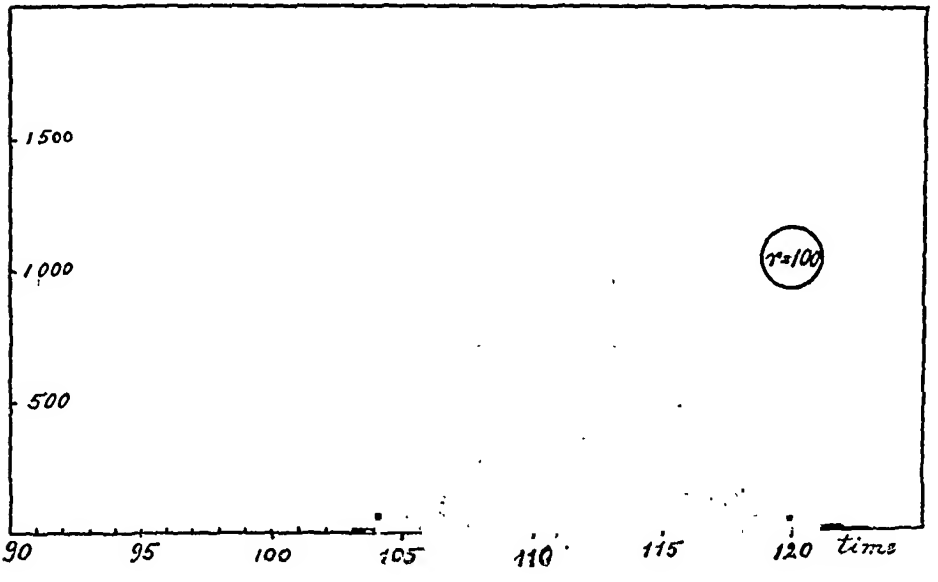


FIG. 6. Deaths per unit time with 100 reacting molecules per cell

except for $r = 1$, and the increase is very noticeable even if we chose as zero time, n_o, not the beginning of the exposure, but the beginning of dying. The larger r, the larger is the increase. This becomes more conspicious if we consider the *relative* "death rates," taking the rate of the first time unit = 100.

As a matter of fact, an increasing death rate has been very rarely observed. Reichenbach's (1911) unknown sporeformer, Chick's Staphylococcus and Myers' Bacillus 25 are probably the only cases on record for an increase of K. If the death rate is not constant, it

usually decreases. The decrease can be easily accounted for by the assumption of a graded resistance, the mass law holding for each grade. The death rate is at first influenced by the less resistant cells, and towards the end of the experiment, when the more sensitive cells are all dead, the resistant survivors show a lower death rate.

This circumstance that the death rate is either constant or decreasing, but very rarely increasing, is another strong argument for the assumption of $r = 1$ in bacteria.

TABLE V

"Death Rates" of the "Theoretical Case of Disinfection"

$$K = \frac{1}{t} \log \frac{a}{b}$$

Time interval	$r = 1$	$r = 2$		$r = 3$		$r = 4$		$r = 10$		
	$t_0 = 0$	$t_0 = 0$	$t_0 = 1$	$t_0 = 0$	$t_0 = 2$	$t_0 = 0$	$t_0 = 3$	Time	$t_0 = 0$	$t_0 = 9$
0-1	1.0	0	—	0	—	0	—	9-10	0.0213	0.213
1-2	1.0	0.361	0.721	0	—	0	—	10-11	0.0526	0.289
2-3	1.0	0 518	0.776	0.189	0.567	0	—	11-12	0 0961	0.384
3-4	1.0	0 608	0.811	0.320	0.641	0.116	0.453	12-13	0.1460	0.473
4-5	1.0	0 667	0.834	0.417	0.689	0.218	0.545	13-14	0.2000	0.557
5-6	1.0	0.706	0.852	0.483	0.724	0.300	0.600	14-15	0.2555	0.639
6-7	—	0 742	0 866	0 537	0.751	0 366	0.641	—	—	—
7-8	—	—	—	0.560	0.773	0 421	0.673	—	—	—
8-9	—	—	—	—	—	0.466	0.699	—	—	—
				Relative Death Rates						
	100		100		100		100			100
	100		108		113		117			136
	100		112		122		130			180
	100		116		128		138			222
	100		118		133		146			262
	100		120		136		151			300

The question may well be asked how the type of curve illustrated in Figs. 5 and 6 will ever come to show that all large organisms behave alike and die at the same moment. The explanation for this involves the time factor.

In the development of the formula upon which this discussion is based, it was implied that, when we had two reacting molecules per

unit, the units were twice as large. Otherwise, it would mean a change of concentration. But in computing Tables III and IV, the same initial number of cells, $a = 1{,}000{,}000$, has been used. The 1,000,000 cells for $r = 12$ contain 12 times as many reacting molecules as the 1,000,000 cells for $r = 1$. This has not been important for our considerations so far, because neither the shapes of the curves nor the death rates would be changed at all if for $r = 2$, the initial number of cells were chosen as 500,000.

But if in one case, we have 10 times as many molecules as in the other, it is certain that it will take more time to kill all the organisms containing each 10 times as many molecules. Our Tables III and IV show this very distinctly. Since the survivor curve approaches zero asymptotically, we might best compare the times required to reduce the number of living cells to less than 1. This requires for $r = 1$ only 6 minutes, for $r = 4$ about 12 minutes, for $r = 11$ about 18 minutes, and for $r = 100$ about 128 minutes. Of this time, a considerable portion passes before any deaths occur at all. This time increases with the number of reacting molecules. It increases not only absolutely, but also relatively. Computing the time of action without death in terms of the total time needed to kill 99.9999 per cent of all organisms, we obtain:

TABLE VI

Time Required to Kill 999,999 Out of 1,000,000 Cells

r	Total time for disinfection	Time before first death		Period of dying	
		Absolute	Per cent of total time	Absolute	Per cent of total time
1	6	0	0	6	100
2	8	1	13	7	87
3	10	2	20	8	80
4	12	3	27	8	73
6	14	5	36	9	64
8	16	7	41	10	59
10	17	9	53	8	47
12	20	11	55	9	45
100	128	99	77	29	23

If the curves of the deaths per unit time are plotted so that the total time required for killing all cells is reduced to the same scale, we obtain

the pictures represented in Fig. 7. The total abscissa presented, 6 time units, is required to reduce 1,000,000 living cells to less than one; this cannot be shown in the curves because 1,000 cells are the smallest number that is barely visible on this scale. The figure illustrates,

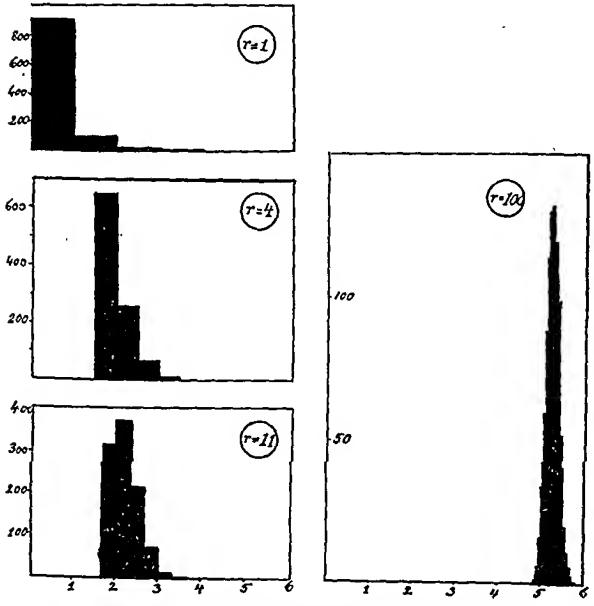


FIG. 7. Deaths per unit time for different numbers of reacting molecules (as in Figs. 5 and 6), but drawn to a standard time scale

however, how, with increasing r, the relative time required for killing becomes less, and how, for $r = 100$, it approaches a line. It is not very likely that such a curve could be obtained by experimental measurement. If we measured even every half minute, we would

find no cells killed until the 9th half minute, 23,000 killed in the 10th half, 947,000 in the 11th half, and 20,000 in the last half. This means practically all cells, (94.7 per cent) killed between 5 and 5.5 minutes. This is a good approximation to the "vitalists'" claim that if all cells are absolutely alike, they should all die at exactly the same moment. It is easily seen that for $r = 200$, or $= 1000$, the approximation would be still better.

There is one other method used by bacteriologists to prove the existence of the mass law in disinfection, *i.e.* if the logarithms of the

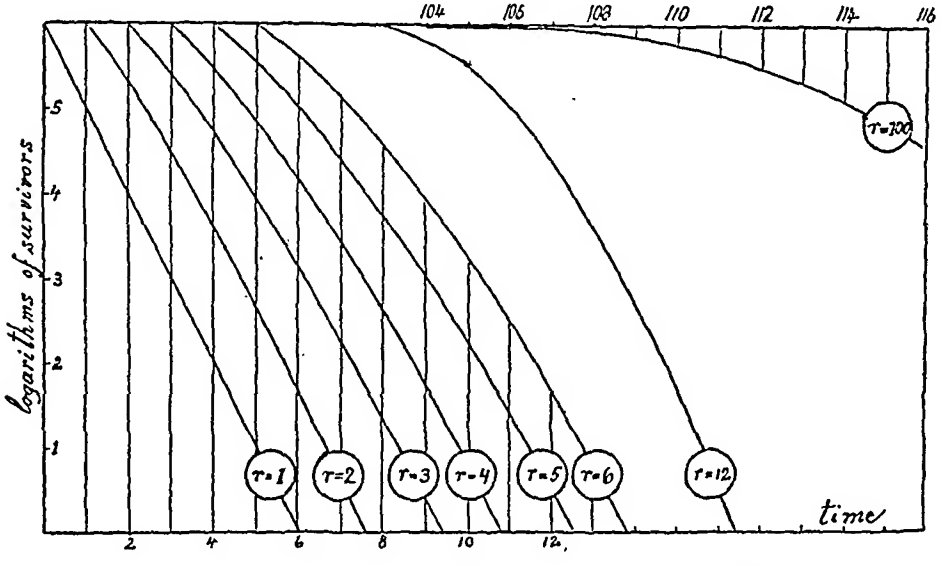


FIG. 8. Time-relationship of the logarithms of survivors, for different numbers of reacting molecules

survivors are plotted against time, they should be on a straight line. If this same method is applied to our data of Table III, we obtain the curves shown in Fig. 8. The curves obtained are not straight lines (except for $r = 1$) but they are near enough to straight lines to be considered as such if we omit the initial number. Unfortunately, many bacteriologists have disregarded the initial number and have started the counting of survivors after the bacteria had been exposed to the unfavorable condition for some time. All data by Paul and his associates on the action of acids upon Staphylococcus, and many data by Chick are of no value on account of this omission. Any reconstruc-

tion or extrapolation would be quite arbitrary. This circumstance reduces the number of proofs for the logarithmic order considerably. If the initial number is included, a straight-line relationship of the survivors is a fairly good criterion for the logarithmic order.

DISCUSSION

The above conclusions and deductions refer to unicellular organisms of exactly the same resistance. No allowance is made for individual variation or graded resistance. If a chance distribution of resistance is assumed, this would mean a superposition over the curves outlined above.

The large number of experiments on the theories of disinfection by heat, chemicals, light, and drying, show, with comparatively few exceptions, an approximation to the logarithmic order. As criterion for the logarithmic order, the constancy of the death rate, $K = \frac{1}{t} ln \frac{a}{b}$ is chosen. This death rate has frequently been found to decrease; this can be accounted for by a superposition of a graded resistance over the mass law equation. The data of Table V show that with more than one reacting molecule per cell, the death rate must increase. This has been rarely found with bacteria. The author believes the evidence to be quite decidedly in favor of the assumption that in bacteria, as a rule, there is a group of special molecules so essential for their existence that the destruction of only one and in some cases perhaps 2 or 3 of these molecules kills the cells. How many molecules of this type exist per cell, we are unable to say.

With multicellular organisms, the logarithmic order does not hold, nor could we possibly expect it to hold. The situation becomes very complicated. Even supposing that all cells are exposed simultaneously to the harmful influence which might be possible with exposure to heat, but not to chemicals, death of the entire organism will begin with death of the individual cells. But the death of one cell does not mean the death of the entire organism. We have here a repetition of the relation between molecules and cells on a higher level; this time, death depends upon the number of cells that must be killed to prevent recovery of the organism. The mathematical treatment of this problem seems possible, but extremely complex.

CONCLUSIONS

Death of unicellular organisms is brought about by the inactivation of a certain number of essential molecules in the cell.

If the number of these essential molecules is only one per cell, the order of death is the same as if the cell were identical with this molecule; the order of death is logarithmic following the mass law.

If more than one molecule must be inactivated before the cell dies, the order of death is not logarithmic. With 2 or 3 molecules, it still resembles the logarithmic order, but with an increasing number of reacting molecules, it approaches more and more the order of death known with higher organisms, namely a period of no death, followed by a comparatively short period of rapid death.

The decision whether or not the logarithmic order exists, should be based upon the constancy of the death rate $K=\frac{1}{t} ln\frac{a}{a-x}$. The existence of a straight line when logarithms of survivors are plotted against time, *is not sufficient proof unless the initial number of cells is included.*

These deductions are made with the assumption that all organisms are exactly alike, and show no individual variations or graded resistance.

With most bacteria, the order of death is so nearly logarithmic that death must be brought about by the inactivation of only one molecule, though there may be several molecules of this same type in each cell.

REFERENCES

Brooks, *J. Gen. Physiol.*, 1919, **1**, 61.
Chick, *J. Hyg.*, 1908, **8**, 92.
Chick, *J. Hyg.*, 1910, **10**, 237.
Chick and Martin, *J. Physiol.*, 1910, **39**, 404; 1911–12, **43**, 25.
Cohen, *J. Bact.*, 1922, **7**, 183.
Falk, *Abstr. Bact.*, 1923, **7**, 33.
Falk and Winslow, *J. Bact.*, 1926, **11**, 1.
Fulmer and Buchanan, *J. Gen. Physiol.*, 1923, **6**, 77.
Hewlett, *Lancet*, 1909, **I**, 741.
Ikeda, *Z. Hyg.*, 1897, **25**, 95.
Lee and Gilbert, *J. Phys. Chem.*, 1918, **22**, 348.
Loeb and Northrop, *J. Biol. Chem.*, 1917, **32**, 103.

Madsen and Nyman, *Z. Hyg.*, 1907, 57, 388.
Paul, *Biochem. Z.*, 1909, 18, 1.
Paul, Bierstein, and Reuss, *Biochem. Z.*, 1910, 25, 367; 29, 201.
Reichenbach, *Z. Hyg.*, 1911, 69, 171.
Reichenbach, *Centr. Bact., I Abt. Orig.*, 1922–23, 89, 15.
Tammann, *Z. physik. Chem.*, 1895, 18, 426.

DISSIMILARITY OF INNER AND OUTER PROTOPLASMIC SURFACES IN VALONIA. II

By E. B. DAMON

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, May 24, 1929)

In an earlier publication[1] from this laboratory experiments have been described in which the P.D. was measured between the inner and outer protoplasmic surfaces of *Valonia macrophysa*.

Electrical connection with the sap of the (very large) vacuole was made by piercing the cell with a fine glass capillary tube filled with artificial sap. It was found that on standing the protoplasm becomes attached to the glass, forming a seal which prevents short circuit between the cell wall and the sap at the spot where the tube passes through the protoplasm. When both the inner and outer surfaces of the protoplasm were in contact with approximately the same solution (natural sap inside and natural or artificial sap outside) a P.D. of from 4 to 38 mv. was observed, the inner surface being positive to the outer. The average of 100 readings was 14.5 mv. An observed P.D. of this magnitude in the apparently symmetrical chain: sap|protoplasm|sap, led to the conclusion that the protoplasm itself is not symmetrical. As a working hypothesis it has been assumed that the protoplasm is made up of three layers, an outer, non-aqueous layer, X, the aqueous main body of the protoplasm, W, and an inner, non-aqueous layer, Y, different from X.

In these experiments, however, we failed to appreciate the effect of sea water wetting a large fraction of the surface of the cell when we measured the P.D. against a second solution applied at one end only. From further experiments with improved technique we have found that the value reported for the P.D. of this system is too low, and also that the P.D. undergoes characteristic changes with time. These new data support the conclusions as to the asymmetric structure of protoplasm which were advanced in the earlier paper.

[1] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, 11, 193.

In nearly all our earlier measurements, the *Valonia* cell hung free from the capillary tube on which it was impaled; in a few cases the cell was also supported from below on a glass ring. In all cases, whether the contact was stationary or flowing, the artificial sap or other external solution was applied to the lower tip of the cell only, while the rest of the surface remained wet with sea water, or with

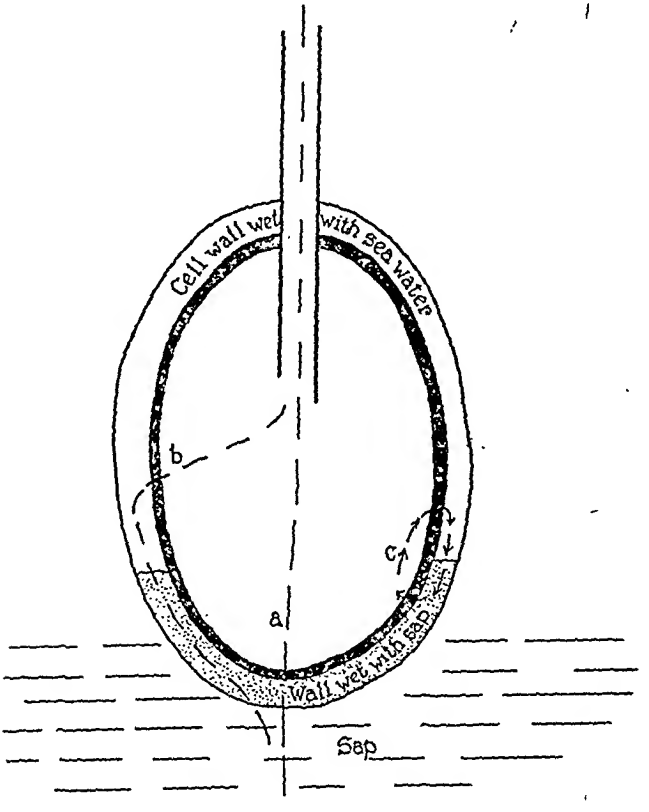


FIG. 1. Diagram of a cell of *Valonia macrophysa* impaled on a capillary, showing conditions when the cell is in contact with more than one solution. At *a* is shown the path along which we desire to measure the P.D.; at *b* is a parallel path in which the E.M.F. across the protoplasm is lower. Differences in the E.M.F. across the protoplasm lead to local currents as illustrated at *c*.

a mixture of sea water and the new solution. Under such conditions we should expect a different E.M.F. across the protoplasm at the upper part of the cell, where the cell wall is imbibed with sea water, from the E.M.F. at the tip, where the protoplasm is in contact with some other solution, such as sap. The observed P.D. will have an intermediate value depending on the relative areas wet by the two solutions. The reason for this is that in addition to the desired path at *a* (Fig. 1),

there are parallel paths, such as *b*, where the E.M.F. is lower. Hence there will be local currents in the direction as represented by the arrows in the path *c*, which will tend to decrease the P.D. against sap, and possibly to increase that against sea water.

These circuits may in a general way be compared to a battery of galvanic cells connected in parallel, where some of the cells are of higher E.M.F. (corresponding to the parts of the protoplasm where sap is applied), and others are of lower E.M.F. (corresponding to the parts of the protoplasm in contact with sea water). Increasing the area in contact with sap is comparable to adding cells of the higher E.M.F., and simultaneously cutting out the same number of cells of the lower E.M.F.

These views were confirmed by experiments in which the P.D. was measured (1) with only the tip of the cell dipping into artificial sap, while the greater part of the surface of the cell remained wet with sea water, and (2) with the entire cell immersed in artificial sap. The following is a typical result:

Tip only in artificial sap, cell wall wet with sea water	8.5 mv.
Entire cell immersed in artificial sap, 2 minutes later	32.6 mv.
Tip only immersed, but cell wall wet with artificial sap, 1 minute later	30 9 mv.

Evidently the P.D. against a given solution applied at one end of the cell may be greatly affected by the presence of a different solution wetting other parts of the cell. Additional evidence of this will be presented later.

In view of this fact, it seemed necessary to carry out further measurements of P.D. in such a way that solutions applied to the cell should wet the entire surface. Also, to prevent contamination by salts leached out of the cell wall, etc., it seemed desirable to apply the solutions by means of a flowing contact such that a steady stream should flow continuously over the entire cell. Our former method of impaling the cell from above was not suited to this procedure, since liquids running down over the cell would be liable to pull it away from the capillary tube, breaking the seal. A better way is to impale the cell on top of a vertical capillary pointing upward; the solutions can then be led on at the top of the cell. Cells so impaled, however, must be supported in such a way that the point of the capillary cannot scratch the protoplasm. This is done conveniently by means of a cork mount as shown in Fig. 2

The lower half of the singly bored cork stopper fits tightly over the glass tube, just below the tip where the tube is drawn out to a capillary. The upper half of the cork is cut away, leaving four prongs, on or in which the cell rests when it has been impaled on the capillary. The complete cell holder, Fig. 3, is a modification of the holder described in our earlier paper. The 250-cc wide-mouth bottle, *C*, is fitted with a two-hole rubber stopper, through which pass the tube on which the cell is impaled, and the funnel tube, *H*. The bottle and tubes are filled with artificial *Valonia* sap. When the cell is impaled, pressure is applied by blowing

in at H, so that the cell is kept turgid while it is pressed down on or within the prongs of the cork mount. After the cell is once in place, the hydrostatic pressure (5 or 6 inches) from the level of solution in H is sufficient to keep the cell distended. Before the impaled cells are used, they are allowed to stand immersed in sea water for two days or longer, to see whether signs of injury appear, and to permit the formation of a good seal.

In measuring P.D. across the protoplasm, contact with the inside of the cell is made through the funnel, H. A string wet with artificial sap leads from H to a beaker of artificial sap into which dips the siphon of a saturated KCl-calomel

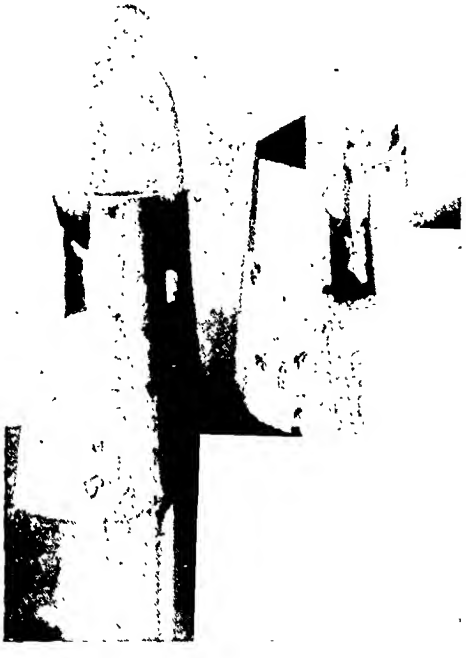


FIG. 2. Photograph showing how the impaled cell is supported by a cork mount.

electrode. Contact with the outside of the cell is made through a strip of wet filter paper touching the highest point of the cell. The solutions applied flow down the filter paper and over the entire surface of the cell. The apparatus used for holding the filter paper is shown in Fig. 3.

The cell is supported over the funnel, F, which catches waste solution and carries it off to the drain. A light platform of paraffin-coated wood resting on the rim of the funnel carries glass rails, r and r', for supporting the strip of filter paper, p, a glass adapter-shaped tube to guide drip from the filter paper well away from the cell, and a cup, c, in which connection is made between the filter paper and the

siphon from a second saturated KCl-calomel electrode. The cup, *c*, has a hole drilled in the side, just under the platform, through which waste solution overflows and drips into the funnel. Solutions from reservoir bottles, flowing down along short lengths of string, *s* and *s'*, are led on the filter paper at the points where it passes over the glass rails, so that a slow stream flows down the paper on both sides. The solution to be applied to the cell is led on the filter paper at *r'*, and molal KCl solution at *r;* contact between the calomel electrode and the solution applied to the cell is thus made through a flowing junction, which should be

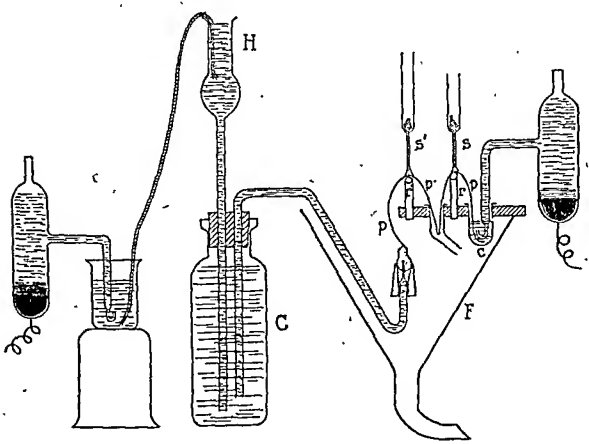


FIG. 3. Apparatus used in measuring the P.D. across the protoplasm of a *Valonia* cell impaled on a capillary filled with artificial sap. The solution applied externally is led on at the top of the cell and flows down over the entire surface.

reproducible, and quickly established when solutions are changed. An advantage of this arrangement is that solutions are changed without opening the circuit, permitting us to obtain an unbroken record of changes in P.D.

Measurements of P.D. reported in this paper, as in the preceding article, were made by means of a Compton quadrant electrometer.

The following experiment, using the new technique of impaling, demonstrates the effect of a second solution wetting a part of the

surface of the cell. The P.D. between the inside of the cell and artificial sap applied to a small spot at one end was measured while most of the remaining surface was wet with a stream of sea water, and again after the sea water had been replaced by a stream of 0.6 M KCl solution.

A rather long cell was impaled as shown in Fig. 4, in such a way that it rested with the long axis nearly horizontal. A strip of filter paper, down which flowed a stream of artificial sap, made contact with one end of the cell at *a*, but brought only a small area in contact with artificial sap. The greater part of the remaining surface of the cell was wet with solution which flowed down a second strip of filter paper touching the top of the cell at *b*. We could then observe the effect of a

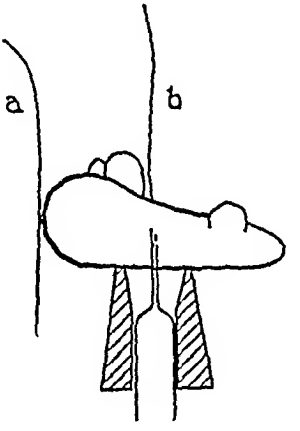


FIG. 4. Arrangement of a *Valonia* cell in an experiment to demonstrate the effect of a second solution wetting a part of the surface.

solution applied at *b* on the P.D. between artificial sap at *a* and the interior of the cell (leading off through the capillary). (Table I.)

As in our earlier paper, the sign of the P.D. is that of the inside of the cell. A positive potential means that the inside of the cell is more positive than the solution applied externally: *i.e.*, positive current tends to flow in the external circuit from the capillary through the electrometer to the solution bathing the outside of the cell.

Obviously, in measurements with *Valonia* (and probably with other marine algae) where the surface must be kept wet with highly conducting solutions, it is undesirable to use methods in which the surface is brought in contact with more than one solution at a time. Experiments of Osterhout and Harris[2] on *Nitella*, however, indicate that this

[2] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 761.

criticism does not apply to measurements on fresh water algae where the surface of the cell is bathed with a solution of high resistance, such as tap water, making the electrical resistance of the cell wall so high, as compared with that of the desired current path, that shunts through the cell wall have little effect. With *Valonia*, it is not practicable to decrease the conductivity of the cell wall by leaching with distilled water, since, except within rather narrow limits, dilute

TABLE I

Time	Description	P.D. at *a* (in contact with artificial sap)
min.		*mv.*
0	Sea water flowing at *b*	9.0
1	" " " " "	8.5
2.5	0.6 M KCl flowing at *b*	24.2
3.5	" " " " " "	23.6
4.5	No solution flowing, surface wet with 0.6 M KCl	20.2
5.5	" " " " " " " " "	19.3
6.5	Sea water flowing at *b*	20.0
8.5	" " " " "	12.8
11.5	" " " " "	8.8
12.5	No solution flowing, surface wet with sea water	9.2
15.5	" " " " " " " "	12.4
17	0.6 M KCl flowing at *b*	22.0

solutions prove highly injurious, even when made isotonic with sea water by the addition of a suitable non-electrolyte such as sugar.

Although Osterhout has observed[3] that *Valonia* cells immersed in their own (natural or artificial) sap live but a short time, generally less than a week, we have found that they easily survive exposure to sap for periods of several hours. Using the apparatus described above, we have followed changes in P.D. against artificial sap applied externally in runs which lasted as long as ten hours.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, 7, 561.

These measurements were carried out at Bermuda during the months of April and May, at room temperatures ranging from 19.5° to 24°C. In most cases, the temperature variation during a single run was less than 1°. In all measurements reported, the cells appeared in good condition at the end of the experiment. They were then returned to a sea water bath and watched for several days; in all cases they continued to look healthy, and gave with sea water the usual P.D. of 5 to 10 mv. Indeed, cells which had been exposed to artificial sap apparently lived longer than others impaled at the same time, and merely kept in sea water, but this may be explained by the fact that the best looking cells were chosen for measurement. The possibility that brief exposure to sap may be beneficial, however, is suggested by the observation of Dr. L. R. Blinks that addition of a small amount of KCl to the sea water in which cells of *Valonia* are immersed assists in keeping the cells alive under unfavorable conditions.

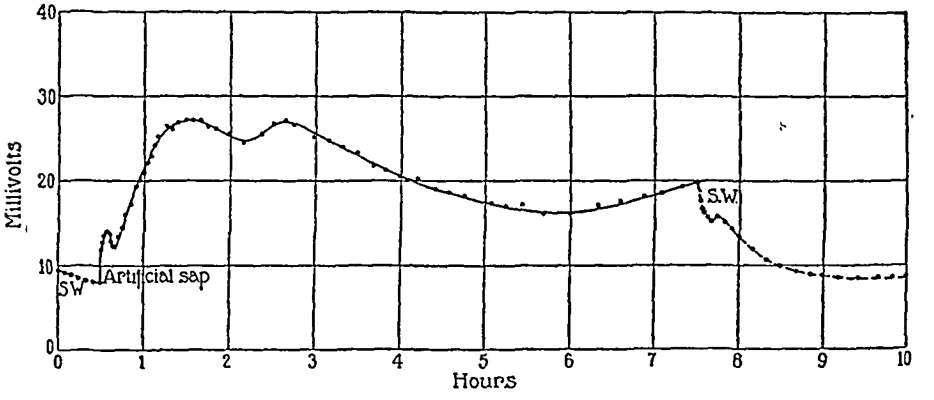


Fig. 5. Curve showing changes in P.D. across *Valonia* protoplasm when the outside of the cell is in contact with artificial sap, and also when the sap applied externally is replaced by sea water.

Results of our experiments are shown graphically in Figs. 5 to 8. It is apparent that the system is changing continuously during the measurement, and that there is accordingly no single value which may be assigned to the P.D. across *Valonia* protoplasm when both surfaces are in contact with *Valonia* sap. In most of the measurements reported here, the maximum P.D. observed was between 25 and 35 mv., but occasionally still higher values are found: an extreme case, when the P.D. rose to 81.8 mv., is shown in Fig. 7.

The graph, Fig. 5, has been selected as a fairly representative curve, but comparison with Fig. 6 (*A*, *B*, and *C*), Figs. 7, and 8*A*, shows that there is a considerable variation in the behavior of different cells. In general, however, we may say

that during the first 90 minutes the P.D. rises to a maximum, falls rapidly to a minimum (which, however, is higher than the P.D. with sea water), then rises slowly to a second maximum. After this, the behavior of different cells is too

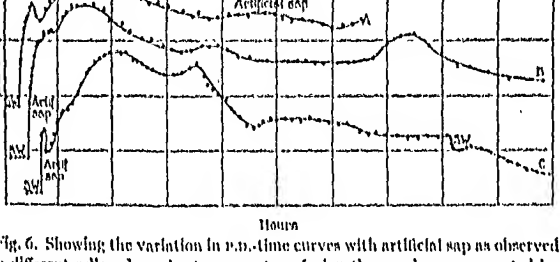


Fig. 6. Showing the variation in P.D.-time curves with artificial sap as observed with different cells. In order to prevent confusion the graphs are separated by vertical displacement (the value in sea water at the start being about 8 millivolts in each case).

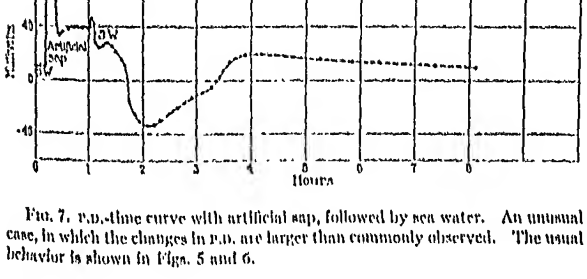


Fig. 7. P.D.-time curve with artificial sap, followed by sea water. An unusual case, in which the changes in P.D. are larger than commonly observed. The usual behavior is shown in Figs. 5 and 6.

varied to permit a single general statement. The first peak is commonly lower than the maximal P.D. observed later, but it may, as in Fig. 7, be much higher; the first and second maxima may be widely separated, as in Fig. 6*A*, or rarely, as in Fig. 6*B*, they may overlap, so that the curve does not pass through a minimum.

In Figs. 5, 6*C*, and 8*B* are shown also the changes in P.D. which are commonly

observed when the cells are returned to sea water (using flowing contact) after they have been exposed to artificial sap. In Fig. 7 is shown a case unusual in that the changes are much greater than those ordinarily found, the P.D. falling to −33.5 mv. (sea water positive); the form of the curve, however, in a general

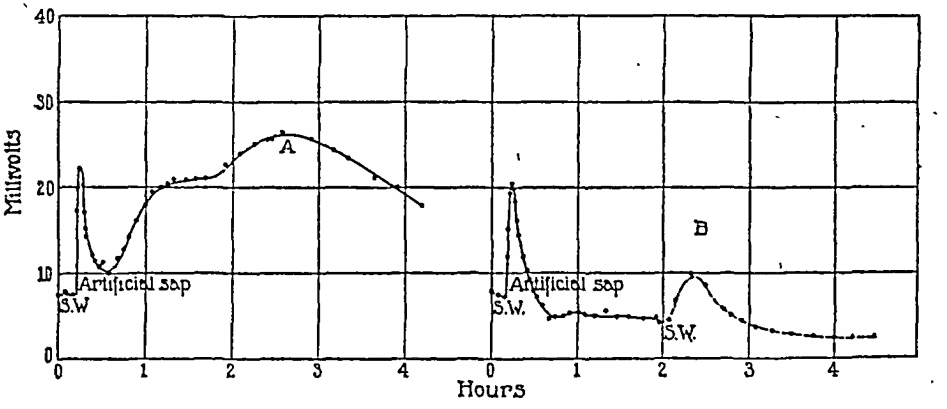


FIG. 8. P.D.-time curves with artificial sap, measured on the same cell, curve *B* fourteen days after curve *A*, showing the change in behavior caused by exposure to artificial sap.

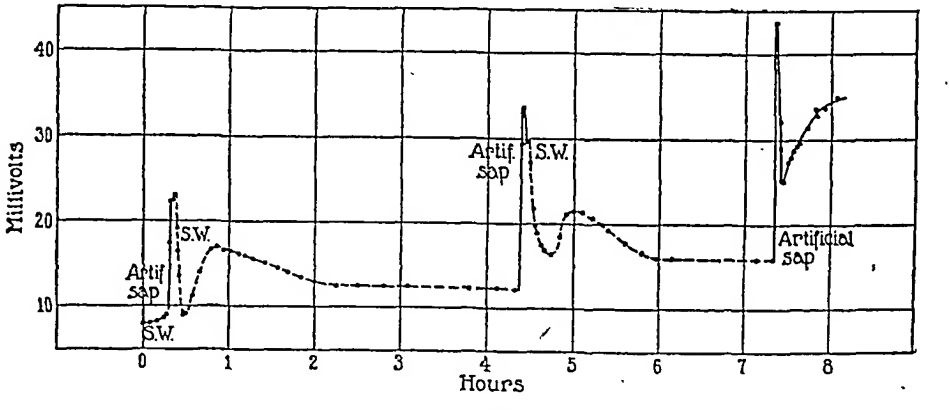


FIG. 9. Curve showing changes in P.D. when the cell is exposed alternately to artificial sap and to sea water, the treatment with artificial sap being too short to produce much alteration.

way resembles the usual one. In most cases, the P.D. at first falls fairly rapidly, passes through a minimum, rises again to a maximum (lower than the P.D. with sap), and then falls slowly and steadily. After a number of hours in sea water, the P.D. becomes more or less constant at a value between 5 and 10 mv., approximately the same as before the experiment.

The appearance of the cells several days after the experiment, and the P.D.'s which they give with sea water, indicate that no permanent injury has been received as a result of exposure to artificial sap. If such cells are used in the second measurement with artificial sap, however, the form of the P.D.-time curve indicates that the cells have undergone an alteration which persists for a long time. This is illustrated by Figs. 8*A* and 8*B*.

The first measurement was made 9 days after impaling the cell; the P.D.-time curve (*A*) is of the usual form: it rises sharply, falls, and rises to a second maximum. After four hours exposure to artificial sap, the cell was returned to a sea water bath. It continued to look healthy, and gave with sea water a P.D. of 7 to 8.5 mv. Fourteen days after the first measurement, a second measurement was carried out in the same way as the first, but with quite different results, as shown by the P.D.-time curve (*B*). The P.D. at first rose to a maximum and fell again as usual, but it then failed to rise to a second maximum. This behavior is typical. The cell was not permanently injured, since, returned to sea water, it continued to live and 10 days later gave with sea water a P.D. of 6.1 mv.

Since prolonged exposure to sap produces an irreversible (or very slowly reversible) change in the protoplasm, it is interesting to compare the effect of brief exposure. An experiment was carried out in which artificial sap was applied for a few minutes only, and replaced by sea water as soon as possible after the beginning of that first fall in potential which presumably indicates that alteration is taking place. Results are shown in Fig. 9. The shape of the P.D.-time curve when sap is replaced by sea water is similar to that which we find after much longer exposure to sap, but the fluctuations are more marked. It is interesting to note that the P.D. rises to a higher value with each successive application of sap, and that after each exposure to sap, the P.D. with sea water returns to a constant level higher than the last. This must indicate that alteration has occurred, but not to the same degree as after long exposure, since when sap was applied for the third time, the P.D.-time curve had approximately the usual shape with two maxima.

DISCUSSION

While it is too early to attempt an explanation of the cause of the P.D. across the protoplasm, it may be worth while to consider what sort of alteration might be expected to lead to changes in P.D. consistent with those which we have observed.

The alteration caused by artificial sap applied externally is probably of a very simple nature, since we can hardly expect the cells to survive a fundamental change in the structure of the protoplasm. Perhaps

the simplest hypothesis is that this alteration consists *merely* in an increased concentration of KCl in the main body of the protoplasm. The characteristic fluctuations in P.D. with artificial sap can be explained, at least qualitatively, as due to such an increase, on the basis of the theory of protoplasmic layers which Osterhout and Harris[4] have found useful in interpreting their results with *Nitella*.

This theory assumes that the protoplasm has an outer, non-aqueous surface layer (X) and an inner, non-aqueous surface layer (Y), different from (X), the two being separated by the aqueous main body of the protoplasm (W). The observed P.D. is then the algebraic sum of the P.D. across X, between the external solution and W, plus the P.D. across Y between W and the vacuolar sap. We assume that the E.M.F. at Y is opposite in sign to that at X and that both these E.M.F.'s are large as compared with their algebraic sum when the external solution is sea water. This assumption is supported, for the case of *Nitella*, by the changes in P.D. observed when the cells are killed by applying solutions saturated with chloroform (current of injury),[4] and for the case of *Valonia* by (unpublished) results of analogous experiments.

These ideas are illustrated by hypothetical diagrams, Fig. 10, where the direction in which positive current tends to flow is indicated by the direction of the arrows, and the relative magnitude of the E.M.F.'s by their length. The observed P.D., the resultant of the E.M.F.'s at X and Y, is shown by feathered arrows.

A represents conditions when the outside of the cell is bathed with sea water, *B*, immediately after the sea water outside the cell, and imbibed in the cell wall, had been replaced by artificial sap. That is, we suppose that the first sharp rise in P.D. occurs entirely at X: this is shown by lengthening the X arrow, and also the feathered arrow which represents the observed P.D. This change in P.D. is no doubt connected with the fact that KCl enters the cell more readily than NaCl, but no attempt is made here to explain the cause of these potentials. They may, for example, be phase-boundary potentials, or they may be diffusion potentials in the non-aqueous phase.

The initial concentration of K salt in W or X is probably low, since increasing the K concentration outside increases the P.D. at X. (If

[4] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, 11, 673.

the K concentration in W were high, an increase in K outside, making conditions at the two surfaces of X more nearly alike, would be

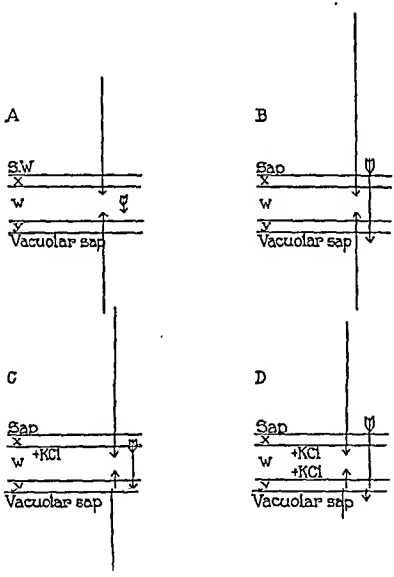


FIG. 10. Hypothetical diagram illustrating the theory of protoplasmic layers. The length of the arrows indicates the relative magnitude of E.M.F.'s assumed to exist across the inner and outer surface layers. The direction of the arrows is that in which positive current tends to flow. The observed resultant P.D. is shown by feathered arrows. *A* represents conditions when the external solution is sea water; *B*, conditions immediately after sea water has been replaced by artificial sap. *C* and *D* illustrate how the penetration of KCl into the protoplasm may cause first a decrease, and then an increase in the observed P.D. (The values represented by the lengths of arrows are fictitious, and do not correspond to any particular experiment.)

expected to decrease the P.D. across this layer.) Now, the entrance of KCl, increasing the concentration in W, will tend to make W ..

nearly like sap, and thus by making conditions on the inner and outer surfaces of X and Y more nearly alike, will tend to decrease the differences in potential across these layers.

The E.M.F. at X will be affected first, before KCl has diffused across W to the boundary of Y. These conditions, illustrated in Fig. 10*C*, are supposed to correspond to the decrease in P.D. which follows the first sharp rise.

As KCl continues to penetrate into W, the P.D. across X will continue to grow smaller, but perhaps at a decreasing rate, since the potential is probably a logarithmic function of the concentration. Meanwhile, diffusion of KCl through W also produces changes at Y. If the rate at which the E.M.F. at Y is affected is greater than the corresponding rate at X, the resultant effect will be an increase in the observed P.D. This is illustrated in Fig. 10*D*, where the arrows at X and Y are both shorter than in Fig. 10*C*, but the feathered arrow, representing the resultant P.D., is longer. This corresponds to the observed rise to a second maximum.

As still more KCl penetrates into W, the E.M.F.'s at both X and Y will fall continuously, but the observed P.D., their resultant, may rise or fall, depending on whether the E.M.F. at X or that at Y changes the more rapidly.

Similar reasoning may be applied to explain the potential changes which are observed when sap is replaced by sea water, since these changes (fall, rise, fall) are the reverse of those (rise, fall, rise) produced by sap. We assume that some KCl comes out of protoplasm, the concentration at X being affected first, that at Y later.

The increase in P.D. observed when sea water and sap are applied alternately (Fig. 9), may be ascribed to a decrease in the E.M.F. at Y, produced by the penetration of a small amount of KCl. Changes at Y are less readily reversed than corresponding changes at X, because of the greater distance of Y from the outer surface.

It is probable, however, that after long exposure to sap a considerable concentration of KCl remains in W, even after the cell has been standing in sea water for several days, and that the absolute values of the E.M.F.'s at X and Y are accordingly much less than with unused cells, although the observed P.D. in sea water, their algebraic sum, may not be different from its usual value. When the cell is measured against sap for a second time, the increase in E.M.F. at X, and hence in

the observed P.D., may be as great as in the first measurement, although the absolute values of the E.M.F.'s at X and Y are much smaller. As KCl penetrates into W, the E.M.F. at X, and with it the observed P.D., will decrease as before. At Y, however, the concentration of KCl is already so great, and the E.M.F. so reduced, that the addition of KCl no longer can decrease the E.M.F. at Y sufficiently to offset the fall in E.M.F. at X. The observed P.D. therefore fails to rise to a second maximum.

SUMMARY

In measurements of P.D. across the protoplasm in single cells, the presence of parallel circuits along the cell wall may cause serious difficulty. This is particularly the case with marine algae, such as *Valonia*, where the cell wall is imbibed with a highly conducting solution (sea water), and hence has low electrical resistance. In potential measurements on such material, it is undesirable to use methods in which the surface of the cell is brought in contact with more than one solution at a time. The effect of a second solution wetting a part of the cell surface is discussed, and demonstrated by experiment.

From further measurements with improved technique, we find that the value previously reported for the P.D. of the chain

Valonia sap | *Valonia* protoplasm | *Valonia* sap

is too low, and also that the P.D. undergoes characteristic changes during experiments lasting several hours. The maximum P.D. observed is usually between 25 and 35 mv., but occasionally higher values (up to 82 mv.) are found.

The appearance of the cells several days after the experiment, and the P.D.'s which they give with sea water, indicate that no permanent injury has been received as a result of exposure to artificial sap. If such cells are used in a second measurement with artificial sap, however, the form of the P.D.-time curve indicates that the cells have undergone an alteration which persists for a long time.

On the basis of the theory of protoplasmic layers, an attempt has been made to explain the observed changes in P.D. with time, assuming that these changes are due to penetration of KCl into the main body of the protoplasm.

PROTOPLASMIC POTENTIALS IN HALICYSTIS

By L. R. BLINKS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, September 14, 1929)

I

The capillary technique used for *Valonia macrophysa*[1] is logically available for other large cells of similar structure. I have recently applied it with success to the closely related *V. ventricosa* of Florida[2] and to *Halicystis*[3] of Bermuda (long confused with *V. ventricosa*[4]). *Halicystis* is a multinucleate cell which superficially resembles *Valonia*, but differs markedly in details of morphology and in the constitution of the vacuolar sap.[5] Its study by the methods developed for *Valonia* is therefore of importance from a comparative standpoint, and its strikingly different behavior must be interpreted in any general bioelectrical theory.

II

Mechanically, *Halicystis* is distinguished by having a more elastic, extensible wall than *Valonia*. The cells are not firm and hard but rather resilient to the touch. They are capable of more shrinkage and swelling without injury to the protoplasm. The tendency to shrink makes the cells more difficult to impale; most of the sap may be lost by spurting through the opening around the capillary before the wound closes. Neither may this loss be made up by sap from the capillary, since hydrostatic pressure through the latter causes a sufficient stream to wash away protoplasm opposite its opening. By the use of sharpened capillaries, however, and by twisting the cells slightly as they are pushed on, most of them promptly form a seal. They then live as long as two or three weeks thus impaled, resting upon cut corks as described by Damon. They may shrink noticeably in

[1] Damon, E. B., *J. Gen. Physiol.*, 1929–30, 13, 207.

[2] Blinks, L. R., *Carnegie Inst. Washington Year Book No. 28*, 1928–29, in press.

[3] Probably *H. ovalis*. A descriptive paper on this organism is in preparation.

[4] Blinks, L. R., *Science*, 1927, 65, 429.

[5] Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, 68, 164.

size during this time, apparently by loss of sap into the capillary. Since the sap of *Halicystis* is less dense than sea water, the larger cells tend to float, and are easily dislodged from the capillary by sudden jar. Cells having a bit of the substrate (calcareous *Lithothamnion*) still adhering to the holdfast remain more surely seated. The most conveniently handled cells are those about 1 cm. in diameter. The capillary may not be pushed far enough into smaller cells to ensure a firm seal, and larger cells tend to collapse on impalement.

The capillaries, drawn on the ends of quarter inch glass tubing, are usually about 0.5 mm. in outside diameter, and from 0.5 to 1.0 cm. in length. They project into the vacuole of the cell 2 or 3 mm. They, and the bottles into which they connect, are filled with artificial *Halicystis* sap made to correspond to the analyses previously published. (The sap of the small cells used in these experiments was

TABLE I

Molar Composition of Saps Expressed as Per Cent of Halide

	A	B	C	D	E
	Sea water Bermuda	*Halicystis* Bermuda	*V. macrophysa* Bermuda	*V. macrophysa* Tortugas	*V. ventricosa* Tortugas
Cl + Br	100.00	100.00	100.00	100.00	100.00
K	2.15	2.58	86.24	82.33	94.74
Na	85.87	92.80	15.08	18.55	5.73
Ca	2.05	1.36	0.288	0.02	Trace
Mg	9.74	2.49	Trace ?	0.08	Trace
SO_4	6.26	Trace ?	Trace ?	0.04	Trace

Analyses: B by Dorcas.
A, C by Van der Pyl.
D, E by Cooper.

essentially the same in composition as that of the large floating cells, according to analysis by Mr. Jacques.) Sodium, potassium, and calcium chlorides are present about as in sea water, with magnesium less concentrated and sulfate absent. (Cells which have formed zoospores and recovered may show sulfate.) The sap thus differs strikingly from that of the *Valonias*, as shown in Table I.[5]

Connection to the outside of the cell was made according to Damon's method,[1] the desired solution flowing down a strip of filter paper in contact with the top of the cell. Certain measurements were likewise made with the cell completely immersed in solution. These P.D.'s were essentially the same as with a flowing contact. Wet strings or salt-agar bridges formed the connection to lead chloride or calomel electrodes. The measuring instruments were a Compton electrometer, and a calibrated vacuum-tube electrometer.

III

Immediately on impalement, the cells of *Halicystis* showed almost no P.D. across the protoplasm. This was the case no matter what solution the cell was bathed in: sea water, artificial sap, or various single salt solutions. The impalement probably caused temporary injury.

On standing in sea water, however, the impaled cells soon displayed a larger and larger P.D. Within 1 hour the value might reach 30 to 40 millivolts, with the *outside positive* to the measuring instrument (*i.e.* positive current tending to flow from outside through the electrometer to the vacuole by way of the capillary; thus the positive current if allowed to flow would be across the protoplasm from inner surface to outer surface). Then more slowly the P.D. continued to rise, usually reaching in a day a maximum which was maintained more or less steadily for as long as two weeks. The highest P.D. found under any condition was 90 millivolts, the lowest steady value in sea water about 50 millivolts. The average value for some 50 cells measured was about 70 millivolts. There was often a fluctuation between 60 and 80 millivolts with the same cell from time to time. All of these were in the direction *outside positive*, and it was not possible to reverse the P.D. by any treatment so far administered.

Exposure of the cells to concentrated and dilute sea water had little of the expected effect on the P.D. A drop of about 10 millivolts was produced by $\frac{1}{2}$ sea water (made isotonic by glycerine) but in one case of long exposure the P.D. returned and rose above the original value; $\frac{1}{4}$ sea water produced a variation of 5 millivolts without permanent effect.

On the contrary, solutions of each of the more important salt constituents of sea water produced an immediate effect, abolishing the P.D. completely. 0.6 M NaCl, 0.6 M KCl, 0.4 M $CaCl_2$, 0.6 M $MgSO_4$, 0.4 M $MgCl_2$, each caused the P.D. to fall to zero in a minute or two, and to remain zero during the exposure. There was occasionally a slight rise just after the preliminary fall, amounting to 5 to 10 millivolts and lasting 2 or 3 minutes (see Fig. 1).

Except $CaCl_2$, which is quite toxic, these solutions did not cause permanent alteration of the cells, even by exposures up to $\frac{1}{2}$ hour.

The P.D. was restored remarkably soon upon re-exposure to sea water. This recovery is shown in Fig. 1 for a typical cell, after exposure to 0.6 M KCl. The recovery was delayed after long exposures but was rapid when once initiated. There was characteristically an "over-shooting" by which the P.D. went to a higher level than before treatment, and

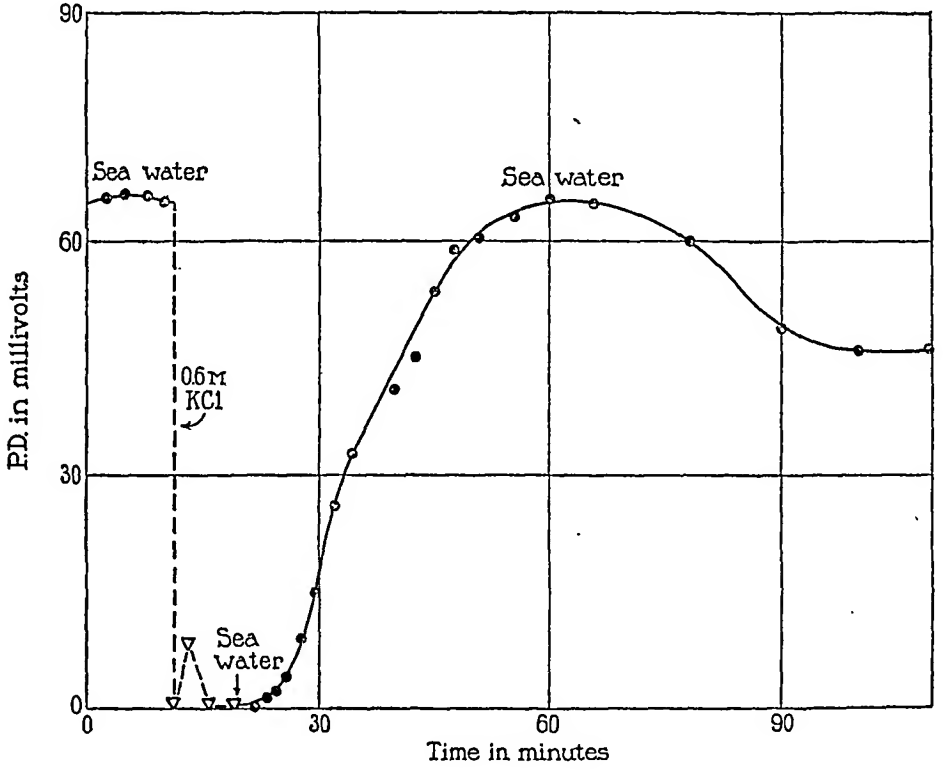


FIG. 1. P.D. in millivolts of an impaled cell of *Halicystis* exposed to 0.6 M KCl; with subsequent recovery in sea water. Time in minutes.

then descended slowly in 1 or 2 hours to a steady value. Occasionally there was only a partial recovery, quickly followed by death.

That the value was dependent on a balanced solution is evident from experiments with mixed salts. Thus the P.D. did not drop to zero when artificial sap was applied to the exterior of the cell, but remained for a long time at about 35 millivolts (Fig. 2). In one case recovery to over 60 millivolts occurred during such exposure. Injury ensued in another experiment, with disappearance of P.D. It is evident that

here again is an example of radial asymmetry in the protoplasm, since similar solutions applied to both sides of the protoplasm still may produce a high P.D. There is apparently a delicate balance at about this composition, since the value with sap is variable.

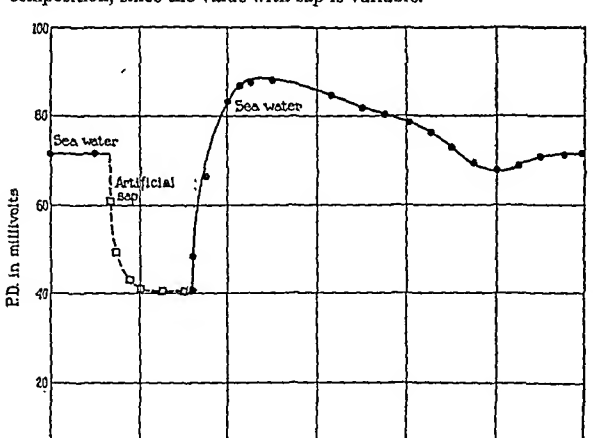


FIG. 2. P.D. in millivolts of an impaled cell of *Halicystis* exposed to artificial sap; with subsequent recovery in sea water. Time in minutes.

The P.D. was still less stable in simpler mixtures, as was exemplified by cells exposed to 0.6 M NaCl, 97.5 parts, + 0.4 M $CaCl_2$, 2.5 parts. The graph of Fig. 3 shows the course of P.D. variation in a cell exposed to this mixture. There is striking evidence here of alternate breakdown and recovery, which suggests the balance of processes dependent not on a single salt, but on several in proper proportion.

IV

It is not possible to draw full theoretical conclusions from the data so far available for *Halicystis*. Two striking facts stand out distinguishing it from *Valonia*.

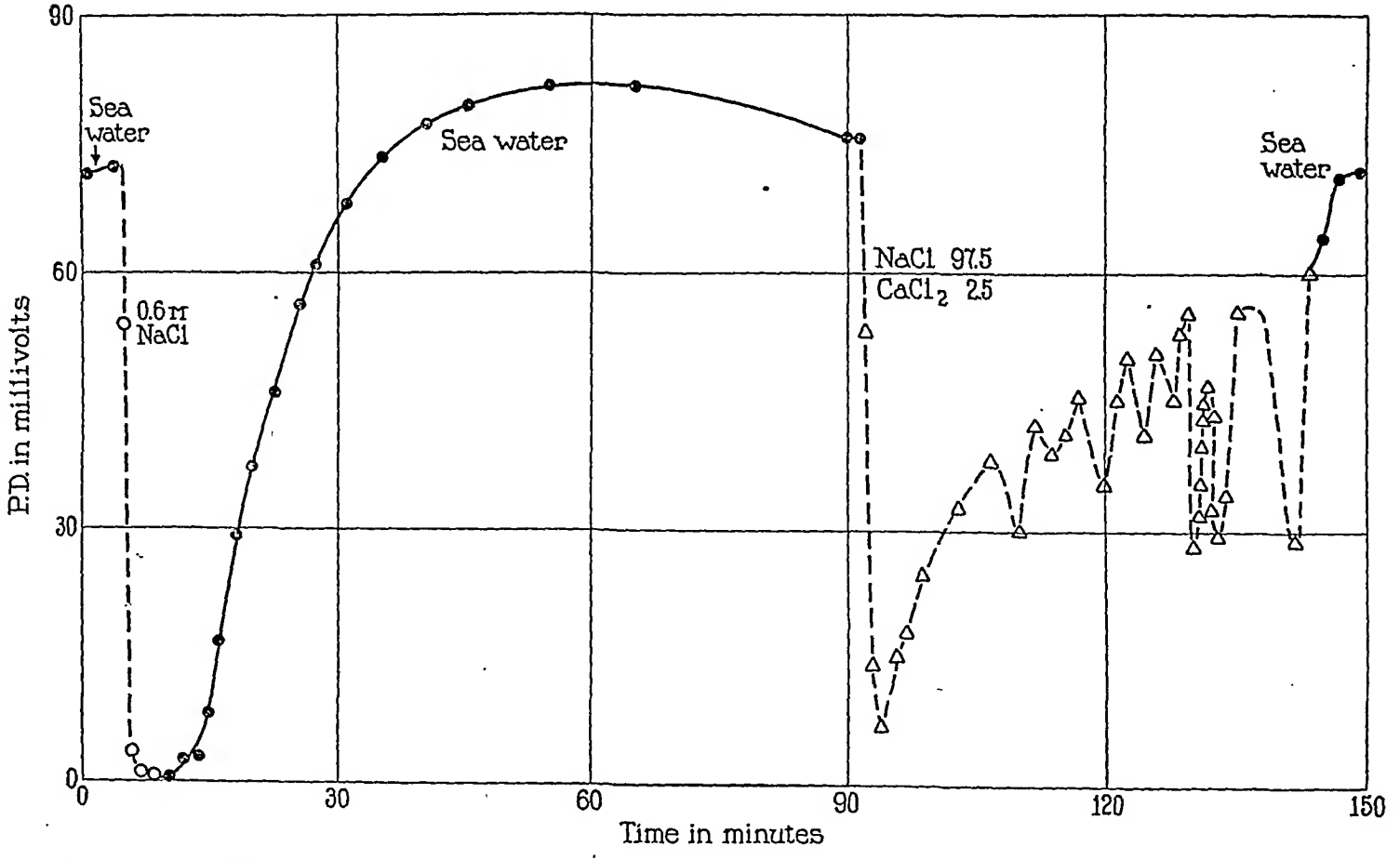


FIG. 3. P.D. in millivolts of an impaled cell of *Halicystis* exposed to (*a*) 0.6 M NaCl; (*b*) a balanced solution of 0.6 M NaCl 97.5 parts, and 0.4 M $CaCl_2$ 2.5 parts; with recovery in sea water. Time in minutes.

1. It shows a P.D. nearly ten times as large as does *V. macrophysa*, and four or five times as large as *V. ventricosa* (both being similarly impaled and immersed in sea water), and directed in the opposite sense. In these respects it is much more like *Nitella* in tap water both as to direction and magnitude of the P.D. The P.D. produced when sap is applied necessarily implies an asymmetric protoplasm.

2. Identification of the ions responsible for the E.M.F. appears difficult in view of the fact that a balanced solution is necessary for the production of any P.D. whatever. Systematic variation of the sea water composition is thus of doubtful value. Except for H^+ and SO_4^- there are no abundant ions of the sea water sufficiently different from those of the sap to give rise to an E.M.F. of 70 to 80 millivolts by concentration effect. That these two are probably not concerned was shown by changing their relative concentration in the sea water. Sulfate ion was doubled by the addition of Na_2SO_4 without effect on the P.D. The pH was changed from 8.2 to 6.0 without immediate effect. (Lower pH produced permanent alteration.)

Further study of these effects will be carried on. It is possible that the slow rise of P.D. observed after impalement is not due to a recovery but to an alteration such as a permanent lowering of E.M.F. at the outer or *X* layer. (This might be produced by the diffusion of salts into or out of the aqueous layer *W*.) Bridge measurements of intact cells show that they have a greater polarization response than the impaled cells, and we have increasing evidence of the expected correlation between polarizability and the bioelectric P.D. It is hoped that the study of *Halicystis* in conjunction with *Valonia* will assist in a general critique of the method of impalement.

SUMMARY

The cells of *Halicystis* impaled on capillaries reach a steady P.D. of 60 to 80 millivolts across the protoplasm from sap to sea water. The outer surface of the protoplasm is positive in the electrometer to the inner surface. The P.D. is reduced by contact with sap and balanced NaCl-$CaCl_2$ mixtures; it is abolished completely in solutions of NaCl, $CaCl_2$, KCl, $MgSO_4$, and $MgCl_2$. There is prompt recovery of P.D. in sea water after these exposures.

A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

I. The Reaction to Monochromatic Radiations

By FREDERICK L. GATES

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, August 4, 1929)

During the past fifty years, many investigators have studied the bactericidal activity of ultra violet light, but a review of their reports shows that little has been learned about many essential factors in the reaction. There have been few contributions to a knowledge of the quantitative relations involved. Little precise information is available on differences in resistance of individual bacteria to monochromatic light and the consequent reaction curve of large numbers of organisms; on the effective range of ultra violet frequencies; on the relation between incident and absorbed energies at various wave lengths; on relations between time and intensity; on the temperature coefficient; on the action of polarized light; or indeed on any of the factors essential to the reaction on which an examination of its biophysical significance must be based.

But the ultimate object of the present study has not been simply to determine a set of coefficients for the bactericidal action of ultra violet light. This particular reaction was chosen for study because less individual variation is to be expected among bacteria than among higher forms of life. They are easily handled in quantity, and the death or survival of microorganisms provides a clearcut endpoint which may be observed and statistically recorded with unusual accuracy for biological material. They seemed to afford the best available opportunity for a quantitative study of an effect of certain frequencies in the ultra violet on protoplasm: with the prospect that the reaction of the bacterial cells might open leads for further studies on higher organisms.

It is obvious that in such work a considerable number of factors

must be controlled, and all but a selected one kept constant under standard conditions, while that one is varied experimentally. In the present study the measured variables were especially wave length, intensity, time, and lethal action, and, except when subject to special experiment, the factors kept as constant as possible were the character and age of the bacterial cultures employed and the composition, temperature, and hydrogen ion concentration of the medium on which the bacteria were exposed and subsequently allowed to multiply.

In quantitative studies energy of a single frequency, or a very narrow range of frequencies is essential: the selective absorption of radiant energy is characteristic of biological materials and a fundamental factor in their reaction to it. Since Grotthus it has been axiomatic that only energy that is absorbed can do work. The exposure of test objects to a whole gamut of radiation frequencies, even when the range is restricted by selective filters, introduces so many unknowns in partial absorption as to void any measurements of the effective energies. The importance of separating the radiations monochromatically has been recognized by Ward (1), Barnard and Morgan (2), Browning and Russ (3), Mashimo (4), and more recently by Bayne-Jones and von der Lingen (5), who exposed bacteria on a nutrient substrate in a quartz spectrograph, and so obtained spectral images of the result. But the equal importance of determining the incident energy required at each frequency to produce comparable effects has apparently been emphasized only by Hertel (6), who reported, however, on only six lines between 210 and 440 mμ. Bang (7) measured the periods of exposure (in seconds) necessary to kill at 20 mμ intervals (with the carbon arc), and tried to correlate the bactericidal effects with bolometric observations on his light source, and Coblentz and Fulton (8) have recently made careful radiometric observations on a few wide regions in the ultra violet defined by filters. But intensity measurements of monochromatic radiations in absolute units, and the correction of incident energies by the absorption coefficient of the objects under test apparently have not been undertaken. Yet it is obvious that physical and chemical analyses of the reaction, and quantitative comparisons with other biological ultra violet reactions can only be made on the basis of such information.

Methods

Light Source. Monochromatic ultra violet radiations were obtained from a vertical, quartz, air-cooled, high intensity mercury vapor arc, with tungsten anode, operating at 67 v., 5.5 amp. direct current. The mercury arc was chosen rather than a spark or a metallic open arc source, because of its steadiness and the intensity and separation of its principal spectral lines. While single frequencies or groups of frequencies within narrow limits are thus easily available, the ultra violet region between wave lengths 2253 and 3126 Å. u. is fairly well-covered by

the radiations at 2302, 2345, 2379, 2482, 2536, 2675, 2804, 2894, 2967, and 3022 Å. u.—all lines, or groups of lines, of sufficient intensity for practical use.

Quartz Monochromator. These specific ultra violet energies were separated and focused by means of a large quartz monochromator of special design.

The vertical quartz mercury lamp is placed on an optical bench, directly behind an adjustable entrance slit, curved to correct the spherical aberration of the quartz optical train. Radiations through the slit are rendered parallel by a crystal quartz planoconvex lens of 75 mm. aperture, and then pass through a Cornu quartz prism of 66 × 41 mm. face, and a large photographic shutter. A second 75 mm. crystal quartz lens then focuses the spectrum on a straight, vertical, adjustable exit slit, by which the desired frequency is isolated and passed to the surface of exposure. The bench supporting the shutter, collimating lens, and exit slit remain stationary, and different wave lengths are thrown upon the slit opening by rotation of the lamp housing, entrance slit, and collecting lens around the axis of the Cornu prism, maintained at minimum deviation by rotation through half the angle. Focus is obtained automatically by a synchronous adjustment of the lamp and entrance slit during their angular travel.

Energy Measurement. The intensity of the monochromatic radiations passed by the exit slit is measured, in the plane of the receiving surface, by thermopiles of special design, patterned after those described by Pfund (9), who kindly gave personal instruction in making them. These compensated, linear thermopiles are of 1.5 × 12 mm. surface, of about 5 seconds period, practically without creep at ordinary room temperatures, and, connected through a ballast resistance with a Leeds and Northrup high sensitivity galvanometer, give readings of about 1 cm. deflection at 2 meters scale distance for a flux of 1 erg mm.$^{-2}$ second (10^{-7} watt), a sensitivity ample for these energy measurements, as the protocols will show. At frequent intervals the thermopile in use was calibrated in absolute units against a Bureau of Standards carbon filament, incandescent lamp, (No. S 26), or a similar secondary standard.*

Test Objects. Although some measurements were made on a laboratory strain of *B. coli communis*, most of the observations here reported were made on a strain of *S. aureus*, originally obtained from Dr. C. G. Bull, and maintained on standard laboratory media for a period of years before these experiments were undertaken. This strain of cocci was chosen because of long adaptation to its environment, and because of the spherical shape of the single organisms, since orientation could not be controlled. During the experiments the strain was transplanted daily on beef infusion peptone 2 per cent agar, buffered at pH 7.4. This same medium was used as the substrate and covering layer in the tests.

For the quantitative determination of a bactericidal effect, it was essential, of

* When energy measurements are given, it should be noted that the energy reported is the total per square millimeter of surface illuminated, and not the energy that falls upon each bacterium.

course, that no absorbing medium should intervene between the measured incident energy and the exposed bacteria. This, and the necessity that all organisms lie approximately in a plane, both during exposure and for subsequent colony counts, precluded the use of a fluid medium, and the following technique was finally adopted.

Experimental Procedure

Small Petri plates, 5.5 cm. in diameter, were attached with wax to 5.1 × 7.6 cm. glass microscope slides and partly filled with a layer of nutrient 2 per cent agar. A carefully made suspension of an 18 hour culture of *S. aureus*, with a transparency limit, or depth-of-disappearance of 30 cm. (10) was washed over the agar surface, the excess drained off, and the plate was allowed to stand in a vertical position until the excess fluid on the surface had evaporated. This method was found to give the most uniform distribution of organisms, so separated that subsequent colony counts could be readily made. The Petri plate was then covered with a crystal quartz plate (to compensate for the quartz window of the thermopile) and the glass slide was set vertically in a mechanical stage, adapted for the purpose, which was rigged on the monochromator so that the agar surface and the thermo-elements could be brought into position alternately, in the same plane, behind the exit slit. The five areas, 4 × 24 mm., to be exposed on each plate, separated by 4 mm. control areas, were then successively located and centered by readings on the mechanical stage. Timed exposures, with monochromatic radiation, and at measured intensities, were made at room temperature, between 20° and 22°C. Tests showed no appreciable variations within this temperature range.

After exposure, the bacteria-strewn surface of the Petri plates was covered with a second layer of nutrient agar at 39°C. In the earlier experiments the use of this covering layer of agar had not been developed, and it was necessary to incubate the plates at 37.5°C. under frequent inspection and to count the colonies in the exposed and control areas as soon as they became clearly visible and before confluent growth occurred. Then it was found that a second layer of agar, flowed on after exposure to cover the bacteria, dislodged an inconsiderable number of them (tens, out of the many thousands on the plate) and that no significant error was introduced by its use. On the contrary, the fixation of the bacteria between two layers of agar practically prevented confluence, and made possible an overnight incubation and a more accurate colony count.

For counting colonies, the exposed and control areas were located under the microscope by corresponding readings on a mechanical stage similar to that on the monochromator. A small central section of each area, measured between parallel lines and between stops on the mechanical stage, and corresponding approximately to the area of the thermopile junctions, was covered in each count.

The experimental errors which this method of estimating bactericidal action involves are obvious. Variations in the house current affect the light source.

Incident energy may not be uniform over the entire surface exposed. Bacteria may occasionally overlie and partly protect one another, and uneven distribution makes appreciable variations in the counts used as controls. This last source of error is the most conspicuous and is largely responsible for the common variations in the results of single exposures in parallel experiments, as shown in Chart 2. If single experiments were used at each wave length, irregular bactericidal curves would result. But each of the curves reported is the average of smoothed curves from a number of parallel experiments, and since errors due to irregular distribution fall indifferently above or below the line, the averages of these smoothed curves approximate closely the true course of the reaction. On the other hand, the use of such smoothed curves (Chart 1) to obtain the average of a number of experiments at each wave length precludes the inclusion of points on the final curves, lest they be interpreted as points of observation, rather than of statistical summary, and thus give a false impression of experimental accuracy. Therefore it will be noted that a number of such curves in this series are reported without points. The alternate method of presentation, when many observations in parallel experiments are scattered irregularly along a common energy gradient, is to collect the points into groups and average them at successive energy levels. For comparison this has been done in Table II, and the results plotted in Chart 2, together with all the single observations and with the curve obtained as the average of smoothed curves from each experiment. The close coincidence of the results of the two methods is apparent and would appear to justify the use of smoothed curves as the more acceptable representation of a continuously progressing reaction.

Before proceeding to an analysis of the experimental results, it may be stated that the effects reported are due to direct action of ultra violet light on the exposed bacteria. Browning and Russ (3), and Coblentz and Fulton (8) reported, and experiments in this series have confirmed the observation that exposure of an uninoculated agar surface to bactericidal wave lengths and intensities of ultra violet light has no measurable effect on the growth of microorganisms subsequently spread on the exposed areas, as compared with adjacent, unexposed controls.

In reports on the bactericidal action of ultra violet light, it is usual to consider first the range of frequencies which are effective. But, as will be shown later, this element in the problem is so intimately related to the specific absorption of energy at different wave lengths that it seems best to defer a consideration of the energies involved at different wave lengths until the typical reaction of bacteria at a single wave length has been examined.

The Reaction to Monochromatic Ultra Violet Light

The first experiments, then, deal with the typical reaction to monochromatic ultra violet light of an 18 hour culture of *S. aureus*, spread on nutrient agar plates, at a temperature of 20°C. and a hydrogen ion concentration of 7.4. The protocol of a single experiment at wave length 266 mμ is given in Table I and the smoothed curve of the findings in Chart 1.

TABLE I

A Single Experiment to Illustrate the Bactericidal Action of Monochromatic Ultra Violet Energy

S. aureus λ266 mμ 7/12/23

Galv. defl. 9.3 cm. Factor: 1 cm. defl. = 1.18 ergs per mm.^{2} sec.

Incident energy = 11 ergs per mm.^{2} sec.

Exp. sec.	2	4	6	8	10	12	16	20	24	30	Controls*	
Energy ergs	22	44	66	88	110	132	176	220	264	330	Plate	
Plate No.	1					2					1	2
Colonies	52	42	38	28	23	20	13	10	2	0	54–67	54–58
Per cent killed	15	31	38	54	62	64	77	82	96	100+	61	56
Plate No.	3					4					3	4
Colonies	70	64	56	43	39	32	19	11	7	0	87–80	78–82
Per cent killed	17	24	32	49	54	60	76	86	91	100+	84	80
Average killed per cent	16	28	35	52	58	62	77	84	94	100+		

* In later experiments control counts were made between each two adjacent exposed areas and averaged in pairs across the plate.

The ends of the curve would have to be obtained by extrapolation, for the least energy used killed some bacteria, and the greatest may have been more than sufficient to kill them all. The general trend of the reaction is to be seen, however, and the curve suggests that for the most part the relation of the incident ultra violet energy to its bactericidal action is logarithmic.

A more complete and accurate curve is obtained by averaging smoothed curves from a number of experiments at the same wave

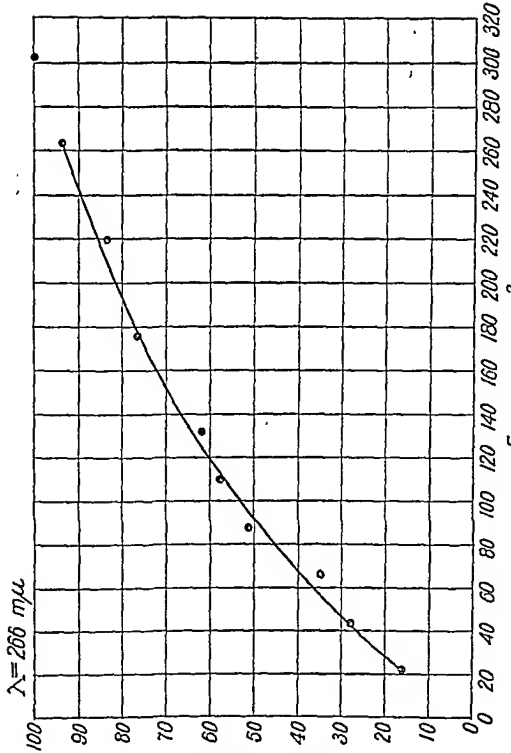


CHART 1. Course of the bactericidal action of monochromatic ultra violet energy in a single experiment.

length, which has been done at each wave length studied. Then the initial and the total bactericidal energies are much more closely approximated, and a curve may be drawn to illustrate the lethal effect from 0 to 100 per cent. Such a curve, the average of smoothed curves from 17 series of observations at λ254 mμ is shown in Chart 2. In this chart all the experimental observations are recorded, and points have been included which were obtained by an alternate method of summarizing these observations, as is shown in Table II. Although approximately the same range of monochromatic energy was covered in each experiment, the separate exposures were timed differently on different days. Hence all the single points in these 17 experiments had to be collected into groups, and averaged at the mid-point of each successive range of incident energies, as indicated.

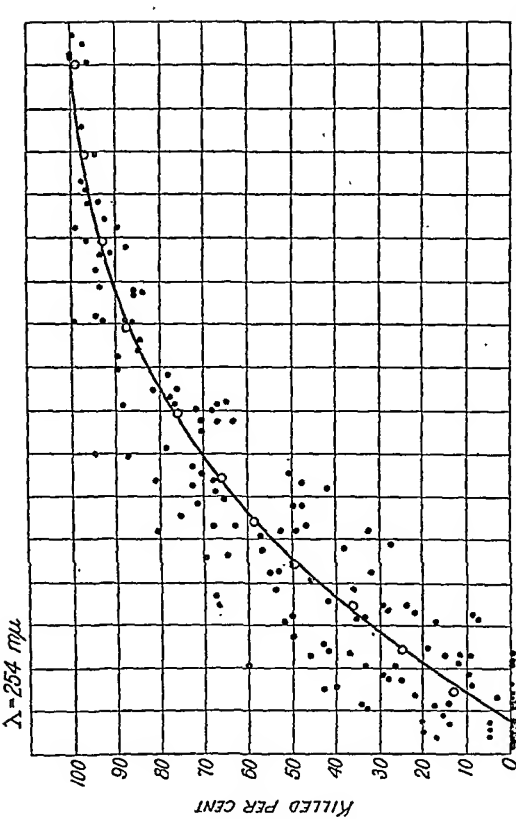
TABLE II

Summary of Observations on the Bactericidal Action of Ultra Violet Energy at λ254 mμ

Energy levels (ergs per mm.²)	20 – 40	40 – 60	60 – 80	80 – 100	100 – 120	120 – 140	140 – 180	180 – 220	220 – 260	260 – 300	300 – 340
Point of average (ergs)	30	50	70	90	110	130	160	200	240	280	320
No. of observations...	22	18	17	11	13	10	17	12	10	4	6
Average per cent killed	13	25	36	50	59	66	76	88	94	97	99

In Chart 3 this curve at λ254 mμ has been included with similar curves obtained at each wave length examined, all drawn with the incident energy recorded on a logarithmic scale. The significance of the fact that very different energies are involved at different wave lengths is reserved for later consideration. But regardless of the absolute energies involved, the curves are so similar as to indicate that the reaction at any one wave length is typical of them all. Each curve shows four successive periods of reaction, clearly seen also in Chart 4 in which all the curves of Chart 3 have been made comparable, and averaged, by expressing the energies, as well as the bacteria killed, in terms of 100 per cent.

1. In an initial period of exposure no bacteria succumb. The energy incident before any bactericidal effect is observed is between 6 and 7 per cent of that required to kill all the organisms.


λ=254 mμ
KILLED PER CENT
0
10
20
30
40
50
60
70
80
90
100
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
ERGS PER mm.²

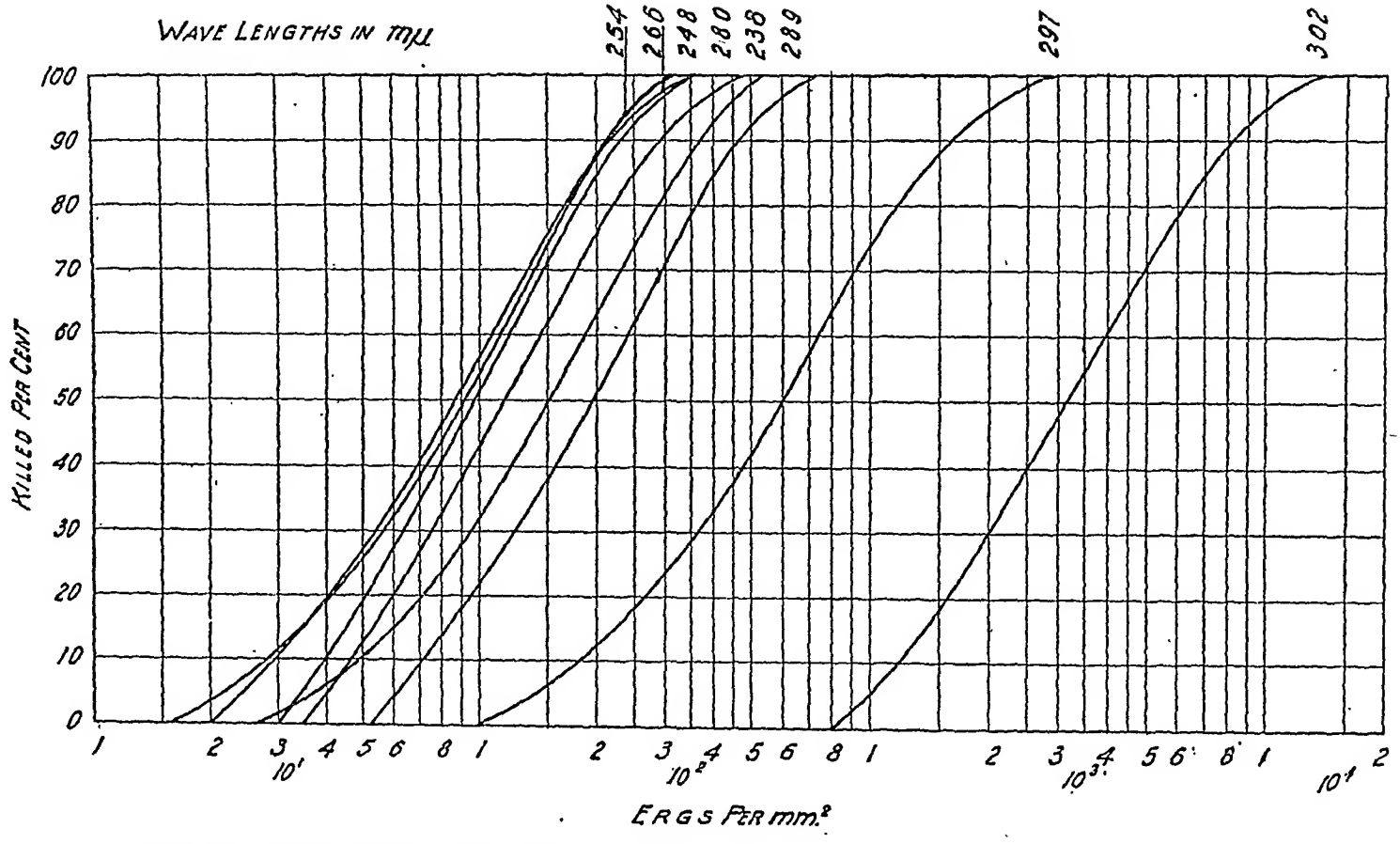


CHART 3. Incident energies required for bactericidal action at various wave lengths in the ultra violet.

2. After this initial exposure a considerable number of bacteria, between 20 and 30 per cent, are destroyed by less ultra violet energy than would be predicted from the rate of destruction for the remainder of the group. They seem to be less resistant than the rest.

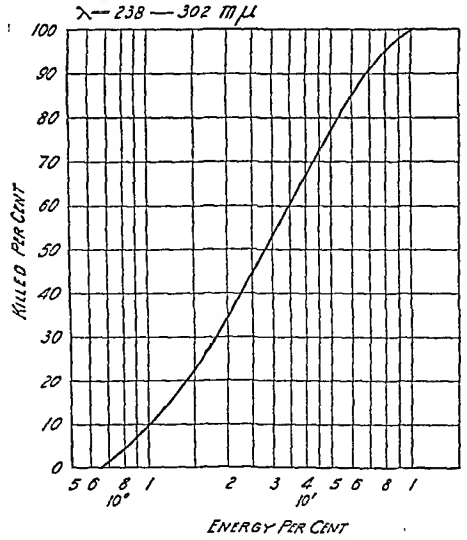


CHART 4. Smooth curve for all wave lengths shown in Chart 3, averaged by figuring the different incident energies from 0 to 100 per cent.

3. Then a considerable number of the remaining bacteria, to about 70 or 80 per cent of the total number, succumb along an energy gradient that appears to bear an exponential relationship to its lethal effect. In Charts 3 and 4, this section of the curves is a straight line.

4. In the final period a number of organisms remain which require an excess of energy to kill them.

Before the significance of the curve, as a whole, can be discussed,

consideration must be given to a number of factors that obviously affect it.

The presence of an initial period of exposure from the effects of which no bacteria succumb was attested by every experiment in which small energies were used. The error introduced by variations in distribution hindered the attempt to determine the initial energies involved in single experiments, so the effect of small energies was subjected to special investigation.

In an experiment at λ254 mμ, 23 plates of *S. aureus* were exposed to small energies, from 7.6 to 32.8 ergs per sq. mm., and it was found that the counts approached the control figures within the common limit of error, and no progressive action was evident in this experiment, until the 32.8 ergs exposure was reached. Then 18.2 per cent of the exposed bacteria failed to multiply.

The fact that bacteria must be exposed to an appreciable ultra violet energy before any of them are killed is evidence that a summation of reactions is involved which finally results in the death of the organisms. But since all the bacteria are exposed to an equal energy, this initial summation effect does not explain why certain organisms are the first to succumb, or why the organisms are not all killed by the same total energy.

The observation that the first 20 to 30 per cent of the cocci succumbed to unduly small energies, but at an increasing rate, until an exponential energy relationship was established, seems to indicate a special susceptibility. And the apparently increased resistance of the last 10 per cent to be killed also suggests that the age and relative resistance of individual bacteria in an 18 hour culture must be taken into consideration. An agar slant culture of *S. aureus*, incubated at 37.5°C., for 18 hours, has not yet reached the limit of multiplication, and contains many organisms but recently divided and in an active metabolic state. It is known that young bacteria are less resistant to ultra violet light than are older, resting organisms (11), and the following experiment shows the greater susceptibility of young individuals in the strain of *S. aureus* under test.

Plates were seeded with distilled water suspensions of broth cultures of *S. aureus*, grown at 37.5°C. for 4, 28, and 52 hours respectively, and were exposed to λ254 mμ. No differences could be detected in the

morphology of colonies which subsequently grew out in the various exposed and control areas, but Chart 5 (from smoothed curves) shows that the recently divided and genetically and metabolically active

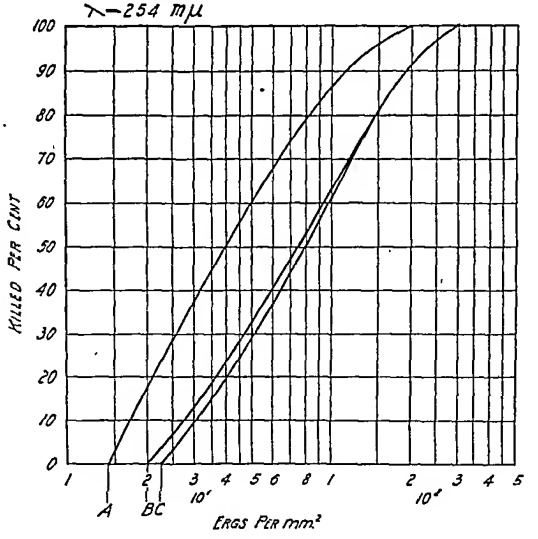


CHART 5. Differences in the course of the bactericidal reaction due to differences in age of the cultures employed.
A = 4 hrs. old.
B = 28 " "
C = 52 " "

bacteria in the 4 hour cultures were appreciably less resistant to the ultra violet energy. This relation between genetic activity and susceptibility to ultra violet radiation will be discussed in the final paper of this study.

It is interesting to note that the incident energy involved in the destruction of the 4 hour culture, in which most of the organisms would

fall in the same genetically active age group, apparently increases logarithmically from the beginning. This is additional evidence that the initial deaths in older cultures are of young and especially susceptible organisms. The cause of the terminal decrease in the rate of destruction cannot be stated with equal assurance. Unduly long exposures were required to kill the last survivors from each culture. But it is not certain whether, due to age or metabolic condition, these bacteria were individually more resistant, or whether they were partially protected in some way from the light. It is possible that small clumps of organisms may persist in the suspensions used, so that some bacteria are overlaid and partially protected. But microscopic slide preparations of many of the suspensions have failed to disclose clumps large enough to afford such protection, and, on the other hand, cocci from colonies of the last surviving organisms have proved to be inherently no more than normally resistant to ultra violet light.

Here may be mentioned an observation which has sometimes led investigators astray. If plates are thickly stewn with bacteria, the resulting colonies are smaller than when nutrient substances are not so limited per colony, and waste products do not accumulate so fast. When few bacteria survive in the middle of an exposed area, or when relatively few are left at the edges, where the intensity falls away, the colonies they produce are always much larger than those in the more crowded control areas of the plate. This has been interpreted to indicate a stimulating action of the light on exposed organisms that were not actually killed (3). But it is only necessary to dilute the original suspension and plate it out on the same medium, so that unexposed organisms are spaced as widely as the survivors in exposed areas are, to determine the source of the more active multiplication and consequent large colonies. Coblentz and Fulton (8) have noted the large colonies from surviving bacteria and have given this explanation of their development.

These experiments, therefore, give no evidence of a stimulating effect of ultra violet light in sublethal doses on the subsequent multiplication of bacteria. They show that there must be a summation of reactions due to radiant energy before any organisms succumb, and that the bacteria of an 18 hour culture show individual variations in their resistance to monochromatic ultra violet light.

DISCUSSION

The characteristic shape of the empirical curve for the bactericidal action of ultra violet light on *S. aureus* (Chart 4) invites an inquiry as to its significance. For it is another example to add to a growing list of experimental biological reactions in which, during most of its course, the reaction rate seems to depend upon the number of reacting units present at the given time.

It is typical of these reactions, such as the killing of bacteria by disinfectants (12, 13, 14), the limitation of the duration of life of fruit flies by untoward conditions of environment (15), or the hemolysis of erythrocytes by specific antibodies, or by ultra violet light (16) that the middle portion of the experimental curve may be reproduced mathematically by the equation that also describes the course of monomolecular chemical reactions. The formula tempts one to speculate, by analogy, on the nature of the fundamental biological reactions involved. But it is equally typical, and important, that toward the ends of the reaction curve the monomolecular reaction formula does not hold good. For example, in the reaction under discussion, there is an initial period of exposure, and consequent summation of its first effects, before any bactericidal action becomes apparent, and after the beginning of the reaction the reaction rate lags for a time before its maximum velocity is attained, although at first the maximum number of cells is exposed to the ultra violet energy. As the surviving units become relatively few, the velocity of the reaction again drops below prediction, and an excess of energy is required before the final bacteria succumb. The characteristic shape of these experimental curves for various biological reactions, with its similarity to the monomolecular reaction curve, and its equally essential differences, has given rise to extended discussions of its significance. It is generally recognized that differences in resistance must and do occur in heterogenous groups of biological units, such as fruit flies or bacteria, or erythrocytes, *in vitro*. If these differences in resistance are essential factors in the reaction of the individual, they must essentially modify the course of the reaction of the group. Under such circumstances, the course of the reaction depends on the distribution among the units of the factors causing resistance, and so the rate is

determined by probability rather than by the fundamental character of the reaction, and the appropriate curve with which to compare the rate is the "mortality curve" of insurance statistics (Chart 6). Then

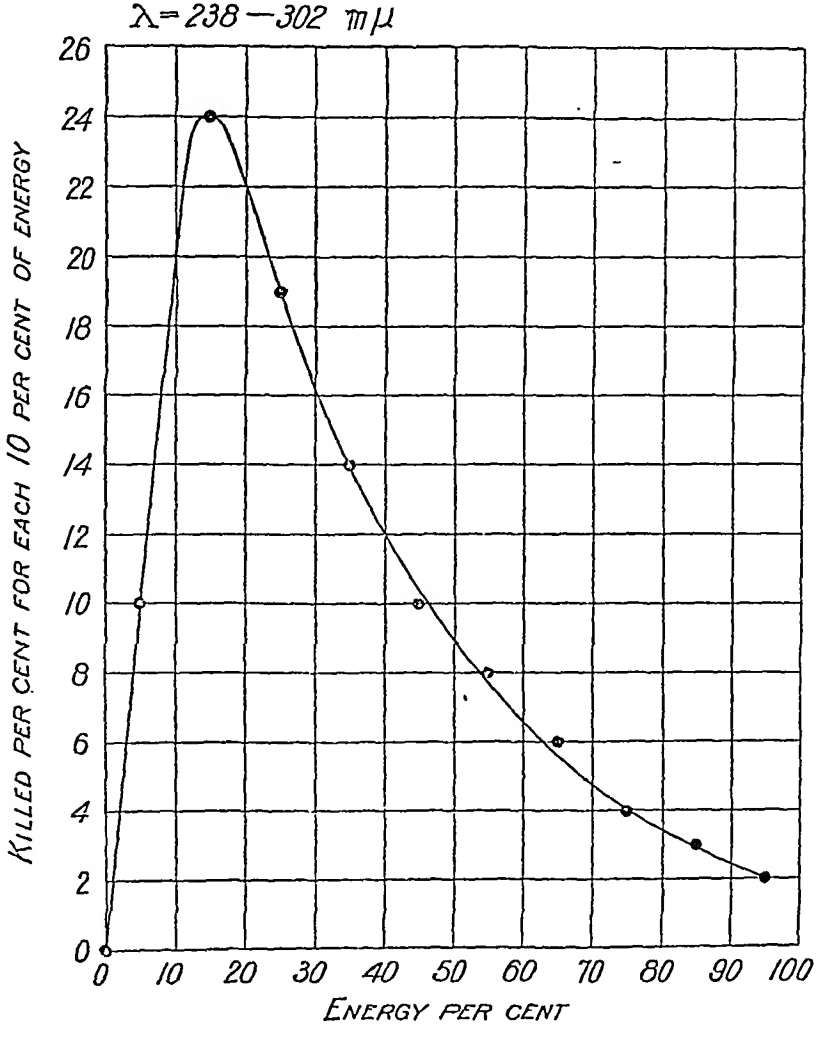


CHART 6. The data of Chart 4 refigured as a curve of probability.

the similarity of its middle portion to the course of a monomolecular reaction is to be ascribed to coincidence within the limits of experimental error—a distribution of resistance factors that simulates a logarithmic curve (15). On the other hand, proponents of the mono-

molecular reaction hypothesis insist that individual resistance varies only within relatively narrow limits, and when large numbers of units are under observation the differences in resistance may be disregarded, while the similarity to a monomolecular reaction persists.

These two opposed points of view are fully reviewed and defended by Brooks (16) and by Cohen (14). At best, the two interpretations affect only the theory of the course of the reactions and throw no light upon their fundamental nature in the various objects under test, as both writers are careful to insist.

The curves for the bactericidal effect of ultra violet light on *S. aureus* are evidently open to either interpretation. Experimental evidence of variations in individual resistance according to age stresses the importance of this factor in determining the course of the reaction. Partial elimination of the age factor by the use of young bacteria produces a reaction curve more nearly logarithmic at the start. But the most that can be said is that the rate of the fundamental reaction, whatever it may be, is undoubtedly modified by variations in individual resistance, and by variations in experimental conditions that mask its true course and make futile any attempt at exact interpretation. And the particular biophysical reactions in each bacterium that result in its death cannot be further analyzed merely from a series of observations on the incident energies that are involved at single wave lengths. A second essential factor in such an analysis—the relation of the incident energy at each wave length to that absorbed by the exposed bacteria—will be considered in a later paper of the series.

SUMMARY

In this first paper of a series on the bactericidal action of ultra violet light the methods of isolating and measuring monochromatic radiations, of preparing and exposing the bacteria, and of estimating the effects of exposure, are given in detail.

At all the different wave lengths studied the reactions of *S. aureus* followed similar curves, but occurred, at each wave length, at a different energy level. The general similarity of these curves to those for monomolecular reactions provokes a discussion of their signifiance, and emphasis is laid upon variations in susceptibility of individ-

ual organisms, due especially to age and metabolic activity, so that the typical curve seems to be best interpreted as one of probability.

REFERENCES

1. Ward, H. M., *Proc. Roy. Soc., London*, 1893, **54**, 472.
2. Barnard, J. E., and Morgan, H. de R., *Proc. Roy. Soc., London, Series A*, 1903, **72**, 126.
3. Browning, C. H., and Russ, S., *Proc. Roy. Soc., London, Series B*, 1917, **110**, 33.
4. Mashimo, T., *Mem. College of Sci., Kyoto Imp. Univ.*, 1919, **4**, 1.
5. Bayne-Jones, S., and von der Lingen, J. S., *Bull. Johns Hopkins Hosp.*, 1923, **34**, 11.
6. Hertel, E., *Ztschr. f. allg. Physiol.*, 1905, **5**, 95.
7. Bang, S., *Mitt. aus Finsens Med. Lichtinstitut*, 1905, **9**, 164.
8. Coblentz, W. W., and Fulton, H. R., Scientific Papers of the Bureau of Standards, No. 495, 1924, **19**, 641.
9. Pfund, A. H., *Physik Ztschr.*, 1912, **13**, 870.
10. Gates, F. L., *Jour. Exper. Med.*, 1920, **31**, 105.
11. Bang, S., *Mitt. aus Finsens Med. Lichtinstitut*, 1901, **2**, 1.
12. Chick, H., *Jour. Hyg.*, 1908, **8**, 92; 1910, **10**, 237.
13. Phelps, E. B., *Jour. Inf. Dis.*, 1911, **8**, 27.
14. Cohen, B., *Jour. Bact.*, 1922, **7**, 183.
15. Loeb, J., and Northrop, J. H., *Jour. Biol. Chem.*, 1917, **32**, 103.
16. Brooks, S. C., *Jour. Gen. Physiol.*, 1918–19, **1**, 61.

A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

II. The Effect of Various Environmental Factors and Conditions

By FREDERICK L. GATES

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, August 4, 1929)

The first paper of this series (1) dealt with the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic ultra violet energy, and it was shown that the course of the reaction was the same at each wave length studied. A consideration of certain factors, such as age and metabolic activity, in the resistance of individual bacteria gave a partial explanation of the course of the reaction among large numbers of organisms. But very different total incident energies were required at different wave lengths to produce these similar effects and an examination of energy relationships and the spectral limits of the bactericidal region was reserved for later consideration.

Before taking up the relation between incident energy and the coefficient of light absorption at different wave lengths, a relation essential to an analysis of the structural elements in bacteria which are affected by light, and to the nature of the resulting reactions, it seems desirable to estimate the effect on the reaction of various conditions of experiment and certain factors in the environment for which due allowance must be made.

The present paper, therefore, will deal with:

(1) The relation between the intensity of the incident energy and the time required for bacterial destruction (the Bunsen-Roscoe Law). (2) The spectral limits of bactericidal action. (3) The temperature coefficient of the bactericidal reaction. (4) The effect of the hydrogen ion concentration of the substrate. (5) The effect of polarization of the ultra violet radiation.

The Bunsen-Roscoe Law

Within the bactericidal zone examined in these experiments (λ238 to λ302 mμ) widely different incident energies were required at different wave lengths to produce similar effects, and since the available source intensities at these wave lengths differed considerably among themselves, it became necessary to know the effect of different intensities on total energies; *i.e.* to determine the validity for these experiments of the Bunsen-Roscoe reciprocity law of photochemistry (2) that when the product of intensity and exposure-time is constant a constant photochemical reaction results. The law does not hold with exactness in certain reactions, and has been modified by Schwartzschild (3) for photographic blackening. Coblentz and Fulton (4) studying the bactericidal action of ultra violet light and employing source intensities in the ratio of $\frac{1}{1} : \frac{1}{10} : \frac{1}{50}$ found that with low intensities a "proportionate increase in the time of exposure falls short of bringing about an equal killing effect." An intensity reduction to $\frac{1}{50}$ required an increase of × 75 in the exposure time to obtain a comparable reaction. This corresponds to a Schwartzschild exponent for the bactericidal reaction of 1.25. When such low intensities and correspondingly long exposures are used with living test objects like bacteria, the fact that the organisms may undergo metabolic or genetic changes during the exposure period must be taken into account, for such changes might themselves modify the reaction. Such wide differences in intensity as 1 and 50 did not enter into the present study, and Chart 1 illustrates the difference in effect of the extremes of intensity involved.

In the irradiation of *S. aureus at* λ266 mμ six series of plates were exposed to an average intensity of 21.6 ergs per mm.² sec. and four to an intensity of 5.6 ergs per mm.² sec. for proportionately longer periods. The averages of these determinations from smoothed curves of each series of bacterial counts (Chart 1*) show that at the lower incident intensity fewer bacteria were killed during most of the reaction period. But it is also evident that the curves for low and high incident intensity approach each other as the reaction progresses and the total energies involved in complete destruction are the same.

* The absence of points on the curves is explained in the first paper.

The differences in the curves would indicate, as suggested above, that it is the younger, metabolically or genetically active organisms which show the greater differences in response to differences in intensity. Since this difference in response varies continuously during the

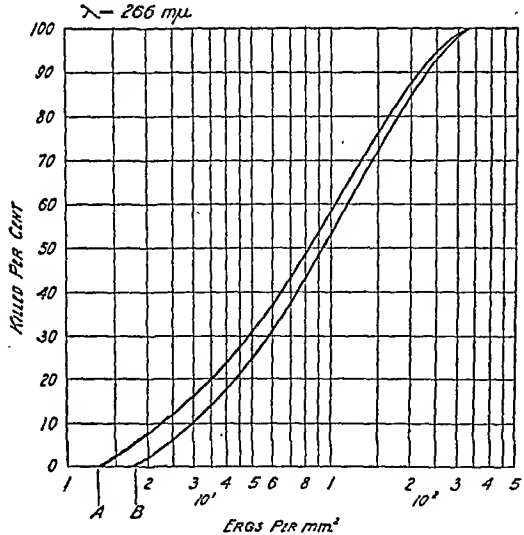


CHART 1. Effect on the bactericidal reaction of different intensities of incident radiation.

A = 21.6 ergs per mm.² sec.
B = 5.6 " " " "

course of the reaction, decreasing as more and more organisms are killed, it is not possible to determine a Schwartzschild exponent except for one point on the curve. Thus at 10 per cent destruction the Schwartzschild modification ($I^q T = K$) of the Bunsen-Roscoe equation requires as an exponent, q = 1.12 while for 50 per cent destruc-

tion differences in intensity must be raised to the 1.08 power in order to determine a time factor that will give the same effect. Except for λ302 mμ the intensities did not differ at the various wave lengths as widely as those illustrated in this experiment, so it is doubtful if greater accuracy would have been attained by adherence to the use of similar intensities at each wave length in the bactericidal range.

The Wave Length Limits of Bactericidal Action

The wave lengths 238mμ and 302mμ noted in the first paper do not define the limits of the bactericidal region of ultra violet light, but simply bound the zone in which complete curves for the lethal action were obtained in the present study. Early observations indicated some bactericidal action at λ313 mμ also, but in later experiments in which stray reflected light was more rigorously excluded even very large energies had no appreciable effect at λ313 mμ. Thirty minute exposures to the 334 mμ and the 366 mμ lines also failed to reduce subsequent colony formation in the exposed areas, and because of other factors, such as metabolic changes in the bacteria, which vitiate quantitative energy determinations with such long exposures, the investigation was not pursued farther in this direction. Other investigators have variously estimated the longer wave length limit of bactericidal action from 295–6 mμ (5, 6) to 350–366 mμ (7, 4) and even into the visible region. Exposures measured in hours (8), however, are of questionable significance, and it seems improbable that in such instances the death of the organisms is due to the direct action of the ultra violet light.

So also with the shorter wave lengths of the far ultra violet, no attempt was made to find and measure a limit to the bactericidal zone. A few experiments with the weak mercury arc lines at λ234, 230, and 225 mμ established only the middle portion of the curves of lethal action, for the low intensities available at these wave lengths prolonged exposures and so increased the difficulty of obtaining accurate results.

Mashimo (5) using an iron spark before a spectrograph in which bacteria were exposed on nutrient agar plates found that with long exposures (150–300 minutes) the limits of bactericidal action on *B. coli* were λ2948–86 and λ1856 Å. u., the limit of air transmission. Lyman (9) had already shown a bactericidal effect of radiations below λ186

mμ and Bovie (10) had extended the lower limit to λ125 mμ by the use of a fluorite window before a hydrogen discharge tube. Although no energy measurements were undertaken he found these Schumann waves highly destructive to protoplasm, so that only short exposures were required for a lethal effect.

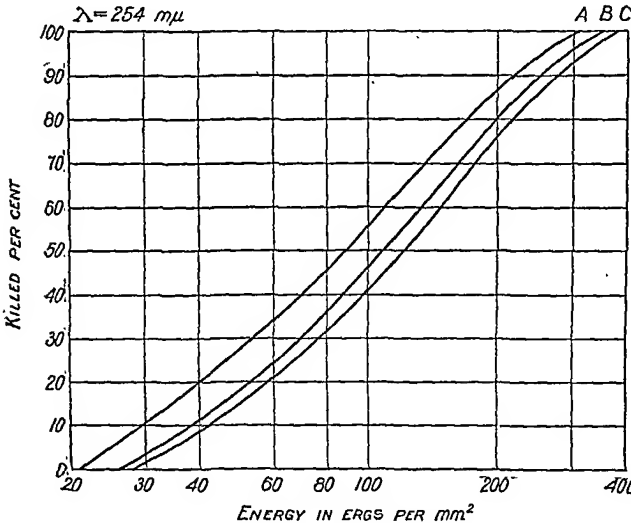


CHART 2. Temperature coefficient of bactericidal action.
A at 36°C.
B at 21°C.
C at 5°C.

The Temperature Coefficient of the Bactericidal Reaction

Of primary importance in determining whether the bactericidal activity of ultra violet light is physical or chemical in nature is the temperature coefficient of the reaction. For although, as Cohen (11) points out, the generalization that the temperature coefficient of physi-

cal reactions is about 1 and of chemical reactions usually above 2 rests on a purely empirical basis, enough experimental evidence has accumulated to warrant a deduction when the observations are clearcut and striking. Even when, in biological experiments, reactions with different temperature coefficients may be progressing simultaneously (12), an observed coefficient for the sum of the reactions which approaches 1 rather than 2 stresses the physical side of the complex. Such was the finding in the present study.

As already stated, most of the exposures were carried out at room temperatures between 20 and 22°C., a range of variation which proved

TABLE I

The Temperature Coefficient of Bactericidal Action

λ 254 mμ

Killed	Energy in ergs per mm.² required		Reciprocal of energy ratio for 31°C.	Reciprocal for 10°C. (temperature coefficient)
	At 5°C.	At 36°C.		
per cent				
20	58	40	1.45	1.13
40	98	70	1.40	1.11
60	148	110	1.35	1.10
80	220	172	1.28	1.08
100	380	316	1.20	1.06
				1.096

to have no demonstrable effect upon the bactericidal reaction. This observation indicated a low temperature coefficient and pointed to a basic reaction physical rather than chemical in nature. But as further evidence, a special series of experiments was made over a much wider temperature range in order to obtain the coefficient for a rise of ten degrees in the environment.

Three series of observations at λ254 mμ were run in parallel, with the agar medium on which the *S. aureus* were strewn maintained at 5°, 21°, or 36°C. during exposure. Thus three groups of bacteria were exposed to the same range of ultra violet energy, but underwent at different temperatures the reactions that resulted in their deaths. The smoothed curves summarizing the experiments are shown in Chart 2, and from these curves the temperature coefficient of the

bactericidal reaction may be figured, for the coefficient would be the reciprocal of the ratio of energies required to produce the same effects at a difference of 10° in the reaction temperature. The ratios of energies involved at 5° and at 36° are shown in Table I, and the reciprocal of these ratios for a change of 10° is found to vary between 1.06 and 1.13, a range of difference within the limits of experimental error. When averaged over the entire course of the reaction, the temperature coefficient is found to be approximately 1.1. Obviously this is the temperature coefficient of a physical (or purely photochemical) rather than a chemical reaction and it suggests that the lethal effect is a direct result of the absorption of ultra violet energy by some essential element, or elements, of the bacterial protoplasm.

The coefficient of 1.06 for the bactericidal reaction when all the organisms are killed (see Table I) is in agreement with the coefficient of 1.05 found by Bayne-Jones and von der Lingen (7) for bacteria exposed in a fluid medium to the total radiations of a zinc spark. They were unable to confirm the reports of Thiele and Wolf (13) and of Wiesner (14) that an increase in temperature extended the bactericidal zone in the direction of longer wave lengths. Cernovodeanu and Henri (8) found no effects of changes in temperature on the bactericidal reaction, but the methods used would not have revealed a coefficient of 1.05 or 1.06.

Hydrogen Ion Concentration

The nearest approach to a variation in the hydrogen ion concentration of the bacterial protoplasm was afforded by exposing the test organisms on agar media of different alkalinities.

The veal-peptone 2 per cent agar medium used uniformly in the series was made up without buffer, and flasks of it were titrated with $\frac{N}{2}$ HCl to pH 4.5 and 6.0, estimated colorimetrically, and with $\frac{N}{2}$ NaOH to pH 7.5, 9.0 and 10.0 respectively. Small Petri plates of these media were then washed with *S. aureus* and exposed in the usual manner to λ266 mμ of the mercury arc spectrum. After exposure all the plates were layered with buffered agar at pH 7.4 and incubated at 37.5°C. overnight. The buffered agar did not bring the hydrogen ion concentration of all the media to 7.4 but to values between 5.5 and 8.2, and within this range no appreciable difference was observed in the number of colonies in equal control ar

Counts of 9 plates at each pH (in 3 parallel experiments) are averaged in Table II and the corresponding smoothed curves are shown

TABLE II

Bacteria Killed, Per Cent, at Different Hydrogen Ion Concentrations

λ 266 mμ

Energy in ergs per mm.²:pH	100	150	200	250	300
4.5	43	63	73	85	95
6.0	44	62	77	89	96
7.5	42	67	77	87	96
Average 4.5, 6, 7.5.....	43	64	76	87	96
9.0	53	71	83	93	99
10.0	52	69	80	89	97

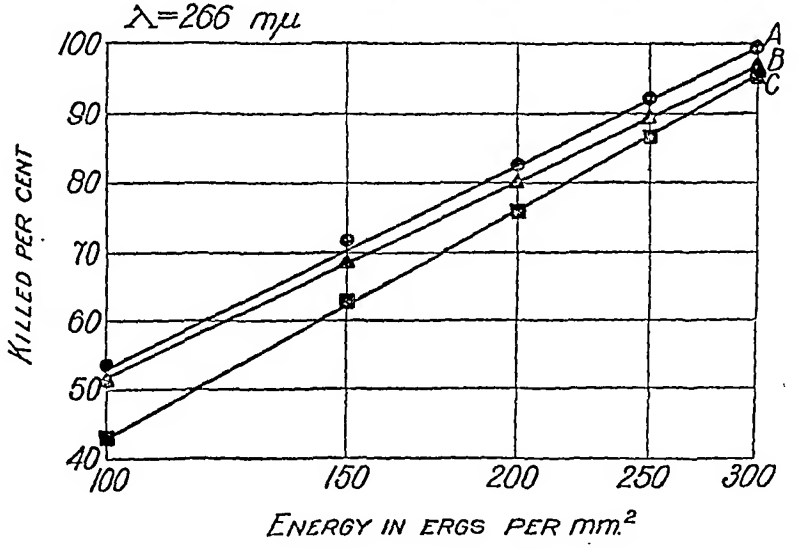


CHART 3. Bacteria killed per cent at various hydrogen ion concentrations of the medium.

A = pH 9.0 ●
B = pH 10.0 ▲
C = pH 4.5, 6.0, and 7.5 (averaged) ■

in Chart 3, except that the points for pH 4.5, 6.0 and 7.5 are so nearly alike that they have been averaged and a single curve drawn through

them in the chart. The figures for pH 9 and 10 approach this average within the accepted limit of error of the method, but since they are uniformly higher, they may indicate a real difference, at these hydrogen ion concentrations, in the susceptibility of *S. aureus* to ultra violet light. Possibly the higher death rates are due to a less favorable environment for the subsequent recovery and multiplication of damaged cells. It is evident that the difference is not so great as to warrant positive deductions to be drawn from these experiments, and one may conclude, on the contrary, that with the methods employed, variations in the hydrogen ion concentration of the substrate between 4.5 and 7.5 would have no appreciable effect upon the bactericidal reaction. Within the range pH 4.5 to 9.0 these results are in accord with those of Bayne-Jones and von der Lingen (7) who found but slight variation in the time required to kill staphylococcus in an alkaline fluid medium. When acid media were used, however, the bactericidal action was greatly accelerated and at pH 2 all the exposed bacteria were killed in 2 seconds exposure, although at pH 6 to 8 the same radiations had required 22 seconds for the same effect.

The Effect of Polarization of the Incident Light

Unless test objects are crystals, or have some plane of symmetry in which they may be placed with respect to a plane of polarization it is difficult to see how polarized light can have a special and significant effect upon them. Yet many examples could be collected from folk lore and from the literature of the alleged biological action of polarized, as distinguished from unpolarized light. This series of experiments afforded an opportunity to determine with measured energies under controlled conditions whether polarization made any difference whatever in the bactericidal action of ultra violet radiation.

After passage through the monochromatic illuminator, the line at 254 mμ was polarized by reflection from a plane quartz surface at the polarizing angle so that no measurable energy traversed a Nicol prism set at 90° to the plane. After removal of the Nicol prism from the path, this polarized monochromatic light was measured in ergs per mm.^{2} sec. by means of the thermocouple and galvanometer, and then used to irradiate *S. aureus* spread on agar plates in the manner already described. Controls were obtained by "depolarizing" the light by a polished plate of crystal quartz. Rotation of a Nicol prism in the beam of depolarized light no

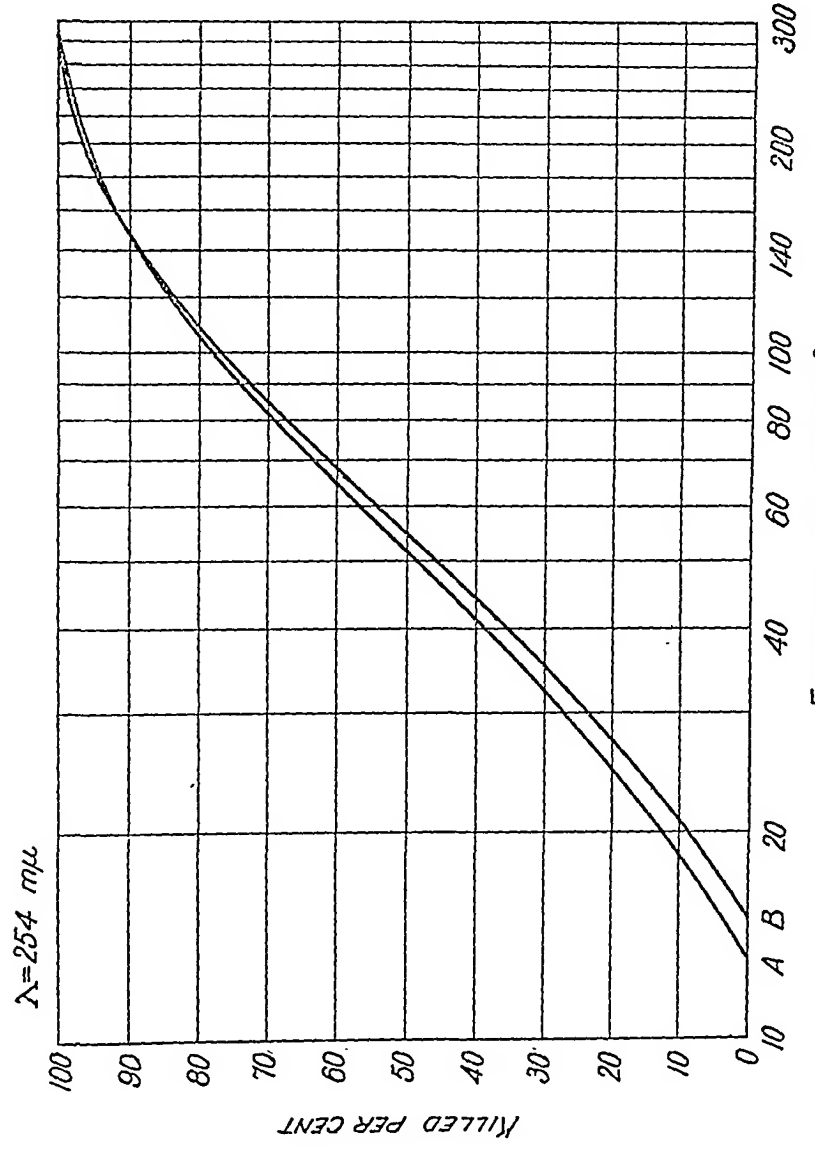


CHART 4. A comparison of the action of (*A*) depolarized and (*B*) polarized ultra violet energy.

longer varied its intensity appreciably. The energy incident on the agar plates was again measured with thermocouple and galvanometer so that a quantitative comparison between the bactericidal action of polarized and depolarized light could be made.

With this set-up eleven series of determinations were made, and the averages are shown in Chart 4. It is obvious that the effects of the polarized and depolarized light are identical, for the differences in the curves are well within the limit of error. That these experiments with polarized and depolarized energy are closely comparable with those done more than two years earlier with unpolarized light (Chart 2, Paper I) is further evidence that plane polarization has no observable effect upon the bactericidal activity of ultra violet radiation.

SUMMARY

1. Wide differences in the intensity of incident ultra violet energy are not accurately compensated by corresponding changes in the exposure time, so that the Bunsen-Roscoe reciprocity law does not hold, strictly, especially for bactericidal action on young, metabolically and genetically active bacteria. In the present series of experiments, however, the energies used at various wave lengths did not differ by so much as to cause a significant error in the reported reactions.

2. The longer wave length limit of a direct bactericidal action on *S. aureus* was found to be between 302 and 313 mμ. The shorter limit was not determined because the long exposures required vitiate quantitative results. Bactericidal action was observed at λ225 mμ.

3. The temperature coefficient of the bactericidal reaction approaches 1 and thus furnishes empirical evidence that the direct action of ultra violet light on bacteria is essentially physical or photochemical in character.

4. The hydrogen ion concentration of the environment has no appreciable effect upon the bactericidal reaction between the limits of pH 4.5 and 7.5. At pH 9 and 10 evidence of a slight but definite increase in bacterial susceptibility was noted, but this difference may have been due to a less favorable environment for subsequent recovery and multiplication of injured organisms.

5. Plane polarization of incident ultra violet radiation has no demonstrable effect upon its bactericidal action.

In a third paper of this group the ratios of incident to absorbed ultra violet energy at various wave lengths and the significance of these relations in an analysis of the bactericidal reaction will be discussed.

REFERENCES

1. Gates, F. L., *J. Gen. Physiol.*, 1928–29, **13**, 231.
2. Bunsen, R., and Roscoe H., *Ann. Physik*, 1862, **117**, 529.
3. Schwartzschild, see: Plotnikow, J., Allgemeine Photochemie, Berlin and Leipsic, 1920, Walter de Gruyter and Co., p. 670.
4. Coblentz, W. W., and Fulton, H. R., *Sci. Papers Bur. Standards*, 1924, **19**, 495.
5. Mashimo, T., *Mem. Coll. Sci., Kyoto, Imp. Univ.*, 1919, **4**, 1.
6. Browning, C. H., and Russ, S., *Proc. Roy. Soc. London, Series B.*, 1917, **110**, 33.
7. Bayne-Jones, S., and von der Lingen, J. S., *Bull. Johns Hopkins Hospital*, 1923, **24**, 11.
8. Cernovodeanu, P., and Henri, C., *C. R. Acad. Sci.*, 1910, **150**, 52.
9. Lyman, T. Spectroscopy of the extreme ultra violet, London, Longmans Green and Co., 2nd edition, 1928, 123.
10. Bovie, W. T., *Botanical Gazette*, 1916, **61**, 1.
11. Cohen, B., *Jour. Bact.*, 1922, **7**, 183.
12. Osterhout, W. J. V., *Jour. Biol. Chem.*, 1917, **32**, 23.
13. Thiele, H., and Wolf, K., *Arch. f. Hygiene*, 1907, **60**, 29.
14. Wiesner, R., *Arch. f. Hygiene*, 1907, **61**, 1.

THE KINETICS OF PENETRATION

I. Equations for the Entrance of Electrolytes

By W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, May 31, 1929)

Difficulties in picturing the mechanism of permeability, and in deciding whether ions can enter clearly indicate the need of mathematical treatment. The brief outline which follows represents merely a preliminary attempt in this direction.

A convenient method of approach is to consider the penetration[1] of a weak acid, HA, into a living cell when it is the only external solute present (conditions being such that penetration of molecules follows a curve of the first order[2]). If we assume that the activity coefficient is 1 we may define the permeability of the protoplasm as equal to the quantity of penetrating substance passing through 1 sq. cm. of protoplasmic surface into the vacuole in unit time under unit concentration (activity) difference.[3]

Case I. Molecules Alone Enter. Let us begin by writing

$$P_M = H_M D_M G_M$$

where P_M is the permeability of the protoplasm for molecules, D_M is the net rate of progress of molecules through the protoplasm,[4]

[1] *Cf.* Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, 26, 192.

[2] E.g., when the inside is stirred by convection currents or by protoplasmic motion and the outside is also stirred. According to J. H. Northrop (private communication) the curve may in some cases be of the first order even if there is no internal stirring, but in that case the time required to diffuse from the surface to the interior of the cell may become the determining factor.

That the penetration may follow a curve of the first order when the sap is stirred by protoplasmic motion is evident from the experiments of Irwin (*J. Gen. Physiol.*, 1925–28, 8, 147).

[3] This formula follows in a general way that of Northrop (*J. Gen. Physiol.*, 1928–29, 12, 435) for penetration through collodion membranes. For molecules we have unit concentration difference when $M_o - M_i = 1$.

[4] This is equal to the reciprocal of the time required to pass through and depends

G_M is a collision factor (such that if one-half of the molecules reaching the surface pass through the protoplasm[5] we may put $G_M = 0.5$); and H_M is a proportionality factor. Let us assume for simplicity that a base *R*OH (produced by the cell) is the only solute of importance inside and that it tends to act as a buffer[6] and yields the

not only on the thickness of the protoplasm but on other factors which may be chemical or physical. In the case of ions the value of D will be intermediate between that of the slower and that of the faster ion.

In case we are considering only penetration through the outer surface into the protoplasm (not into the vacuole) D_M and P_M must be modified accordingly.

If the diffusion gradient be kept constant a steady state will be reached in which the amount entering the outer surface of the protoplasm in unit time will be equal to the amount leaving the inner surface and passing into the vacuole (*cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, 12, 435). We assume for convenience that the protoplasmic layer is so thin that we may neglect the time necessary to reach the steady state (but no actual steady state is assumed for the cases discussed in this paper since the diffusion gradient is not constant).

[5] The factor G is intended to cover the situation regardless of whether the penetration depends on passage through pores, on chemical combination, or on solubility (*cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, 12, 435). The presence of pores in the surface of the protoplasm would seem less probable since the rounding up of drops of protoplasm in water indicates that the surface acts like a liquid but it is not impossible that a thin film (solid or gel) might overlie the liquid (as in the case of a drop of mercury covered with a solid film). In that case water-soluble substances might pass through the pores and lipoid-soluble substances through the substance surrounding the pores.

At any rate we must consider penetration through more than one phase since it would appear that protoplasm may contain several layers. (*Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, 11, 391; also Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, 11, 193.)

[6] In this paper we shall neglect any change of internal pH produced by the entrance of small amounts of H*A* or Na*A* unless otherwise stated. As an example of what is meant let us assume that at the start $M_o = 10^{-4}$ mols per liter, $A_o = 10^{-8}$, $H_o = 10^{-8}$, $ROH = 10^{0.00432}$, $R = 10^{-3.99784}$, $OH_i = 10^{-3.99784}$, $H_i = 10^{-10.00216}$ and $M_i = A_i = 0$, the corresponding values at equilibrium being $M_{oe} = 10^{-4}$, $A_{oe} = 10^{-8}$, $H_{oe} = 10^{-8}$, $ROH_e = 10^{0.004319}$, $R_e = 10^{-3.99568}$, $OH_{ie} = 10^{-4}$, $H_{ie} = 10^{-10}$, $M_{ie} = 10^{-4}$ and $A_{ie} = 10^{-6}$. We assume that the dissociation constant K_B for $ROH = 10^{-8}$ and for $HA = 10^{-12} = K$. These figures satisfy the conditions for a Donnan equilibrium since $(H_{oe})(A_{oe}) = (H_{ie})(A_{ie})$: also $R_e + H_{ie} = A_{ie} + OH_{ie}$. It may be noted that there is little change in pH value due to the entrance of H*A* (*i.e.*, the change is from $H_{ie} = 10^{-10.00216}$ to $H_{ie} = 10^{-10}$).

cation R^+ which is unable to pass out. If the dissociation constant of HA is K we have $(H_o)(A_o) = KM_o$ and $(H_i)(A_i) = KM_i$, where the subscript o denotes outside and i inside, M denotes the concentration (activity[7]) of molecules, and H and A that of the ions. If we may assume that the presence of ROH provides the condition for a Donnan equilibrium we may consider that at equilibrium $(H_{oe})(A_{oe}) = (H_{ie})(A_{ie})$ and $M_{oe} = M_{ie}$, where the subscript e denotes concentration (activity[7]) at equilibrium.

We assume that the internal volume is 1 liter[8] and that the outside concentration is kept constant.[6] Hence at the beginning (when $M_i = 0$) if $M_o = 1000$ millimols per liter and $P_M = 0.01$ the amount entering in the first unit of time is $P_M M_o$ and if we assume for convenience that nothing passes out during the first unit of time the concentration at the end of the first unit of time is $P_M M_o$ or 10 millimols per liter. How much of this remains in molecular form after entering depends on the internal pH value which determines the fractional concentration, F_M, of molecules inside.[9] We may put $F_M = \frac{M_i}{M_i + A_i}$ and since $A_i = \frac{KM_i}{H_i}$ we have

$$F_M = \frac{M_i}{M_i + \frac{KM_i}{H_i}} = \frac{1}{1 + \frac{K}{H_i}}$$

The fractional concentration of A^- inside, F_A, is

$$F_A = \frac{A_i}{A_i + M_i} = \frac{A_i}{A_i + \frac{H_i A_i}{K}} = \frac{1}{1 + \frac{H_i}{K}}$$

We shall for convenience assume F_M and F_A to be constant for any one time curve.

[7] In case the activity coefficient does not equal 1 the treatment must be altered accordingly but this involves no difficulty.

[8] *I.e.*, the total internal volume of all the cells taken together is 1 liter.

[9] If the pH value inside falls during the penetration F_M will increase and the velocity constant V_M will become greater since $V_M = P_M F_M$; it is as though the temperature were to be slowly raised during a chemical reaction of the first order. (The same would be true if the pH value rose during the penetration of a base.)

It is evident that the inward diffusion is proportional to P_M and to the difference in concentration (*i.e.*, to $M_o - M_i$),[10] so that if $S_i = M_i + A_i$ the net amount entering in unit time may be regarded as

$$\frac{dS_i}{dt} = P_M(M_o - M_i)$$

It may be more convenient in the subsequent discussion to assume that there is an inward diffusion proceeding as though M_i and A_i were always zero and an outward diffusion proceeding as though M_o and A_o were always zero. In that case the amount entering in unit time would be $P_M M_o$ and the amount coming out would be $P_M M_i$, so that the net amount entering would be

$$\frac{dS_i}{dt} = P_M M_o - P_M M_i = P_M(M_o - M_i)$$

Since $M_o = M_{ie}$ we have

$$\frac{dS_i}{dt} = P_M(M_{ie} - M_i)$$

where the subscript e denotes concentration at equilibrium. If we multiply the right-hand side of the equation by $\frac{F_M}{F_M}$ we have

$$\frac{dS_i}{dt} = P_M F_M\left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right) = P_M F_M(S_{ie} - S_i)$$

Putting $P_M F_M = V_M$ we have

$$\frac{dS_i}{dt} = V_M(S_{ie} - S_i)$$

and

$$V_M = \frac{1}{t}\ln_e \frac{S_{ie}}{S_{ie} - S_i}$$

We may also put

$$\frac{dA_i}{dt} = \frac{dS_i}{dt}F_A = V_M(F_A S_{ie} - F_A S_i) = V_M(A_{ie} - A_i)$$

---

[10] If a substance must combine with a constituent of the protoplasm in order to enter, the rate of penetration is not a linear function of $M_o - M_i$ but may closely approximate it in many cases. This would also apply to the penetration of ions.

and

$$V_M = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$$

Also[11]

$$\frac{dM_i}{dt} = \frac{dS_i}{dt} P_M = V_M(M_{ie} - M_i)$$

and[12]

$$V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$$

[11] This may be illustrated as follows. If the time unit be sufficiently short we may regard the rate as uniform during any one interval and if at the beginning $M_i = A_i = 0$ we may assume that during the first interval of time the egress is negligible. If $M_o = 1000$ millimols per liter, $P_M = 0.01$, and $F_M = 0.1$ the amount leaving the external solution in the first unit of time is $P_M F_M M_o = (0.01)(1000) = 10$ of which all but 1 is converted into ions after entering so that the increase in M_i is $P_M M_o F_M = (0.01)(1000)(0.1) = 1$. Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$V_M = \frac{1}{t} \ln_e \left(\frac{M_o}{M_o - M_i} \right) = \ln_e \left(\frac{1000}{1000 - 1} \right) = 0.001$$

(whence $P_M = 0.01$). During the next unit of time a part of this escapes by outward diffusion: if we regarded this as $P_M M_i$ we should have $P_M M_i = (0.01)(1) = 0.01$, but when this has passed out we have inside 0.99 M_i and 9 A_i so that $M_i + A_i = 9.99$; but ions must combine until we have $M_i = F_M(M_i + A_i) = (0.1)(9.99) = 0.999$ so that the real loss of $M_i = 1 - 0.999 = 0.001$ or $= P_M M_i F_M = (0.01)(1)(0.1) = 0.001$. The amount of M_i coming in during the second unit of time is, as before, $P_M F_M M_o = 1$. The net increase in M_i in the second unit of time, $\frac{\Delta M_i}{dt}$, is the difference between the incoming and the outgoing quantities or $P_M M_o F_M - P_M M_i F_M = P_M F_M(M_o - M_i) = (0.01)(0.1)(1000 - 1) = 0.999$. Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$\frac{\Delta M_i}{dt} = V_M(M_o - M_i)$$

and for the first two units of time we have

$$V_M = \frac{1}{t} \ln_e \frac{M_o}{M_o - M_i} = \frac{1}{2} \ln_e \frac{1000}{1000 - (1 + 0.999)} = 0.001$$

whence $P_M = 0.01$ (this value is approximate only; the smaller the assumed value of V_M the nearer will be the agreement of the calculated and assumed values).

[12] This equation assumes that the partition coefficient between the protoplasm

This may be checked by considering the condition at equilibrium where we know that the rate of increase of M_i or $\frac{dM_i}{dt} = P_M M_o F_M$ must be equal to the rate of decrease of M_i or $\frac{dM_i}{dt} = P_M M_i F_M$; hence we have $P_M M_o F_M = P_M M_i F_M$: this is correct because at equilibrium $M_o = M_i$.

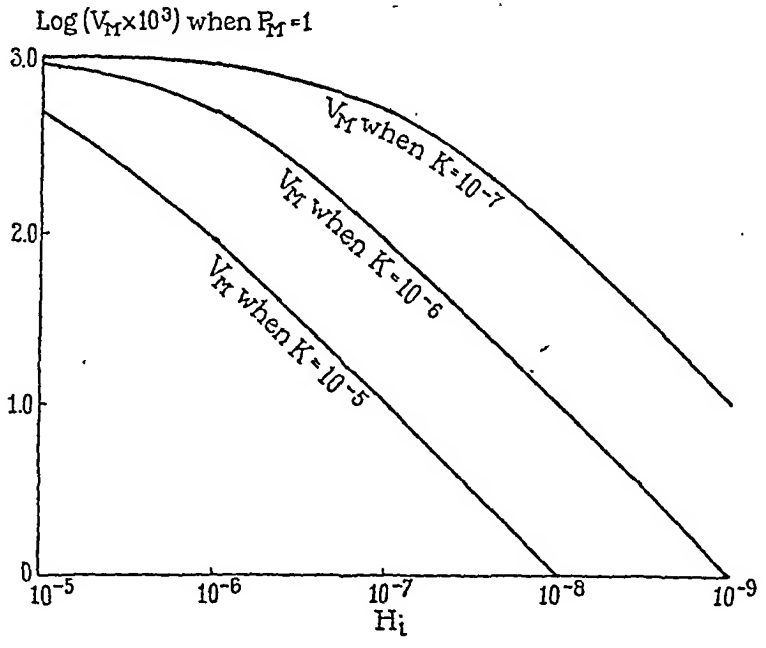


FIG. 1. Shows how V_M ($= P_M F_M$) varies when P_M is constant ($P_M = 1$) but K, H_i (and in consequence F_M) vary. K, H_i and F_M are taken as constant during any one time curve or process of penetration.

In Fig. 1 are shown the magnitudes of V_M for various values of K (the ionization constant of HA) and of internal pH (and hence of F_M) when $P_M = 1$, *i.e.* when $V_M = F_M$.

It should be noted that if M_o remains constant a decrease of F_M (caused by an increase in pH value inside) means an increase of S_{ie} (the total internal concentration at equilibrium, as indicated in Fig. 2) as well as a decrease of V_M.

In order to compare the permeability of the protoplasm for different acids (penetrating as molecules only) we may ascertain the values of V_M and F_M. The latter tends to rise as the acid penetrates,[13] but if the amount penetrating is small and the buffer action of the cell sufficiently great the change in F_M might be negligible. The error would be minimized by taking the rate at the very start (before much change was brought about by penetration), but here the experimental difficulties might make it necessary to construct a

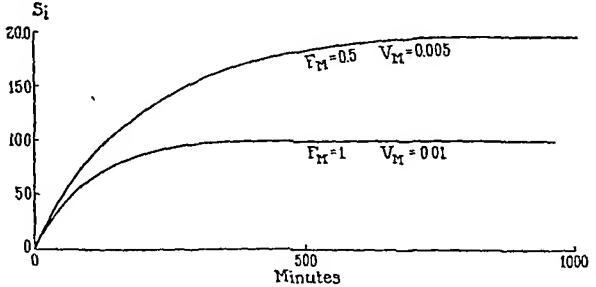


FIG. 2. Calculated time curves of S_i (total amount inside) when molecules alone penetrate and M_o and P_M are constant ($P_M = 1$) but F_M varies and in consequence V_M varies. It is assumed that $M_o = M_{it} = 10$ for both curves. The equation is $V_M = P_M F_M = \frac{1}{t} \ln_e \frac{S_{it}}{S_{it} - S_t}$.

The same curves would serve if ions alone entered by putting V_A in place of V_M and $P_A K = 1$ in place of $P_M = 1$ or if ions and molecules entered by putting V_{MA} in place of V_M and $P_M + P_A K = 1$ in place of $P_M = 1$. In all these cases the rate rises as the external pH value decreases.

portion of the time curve and extrapolate to zero time. At the start, when $M_i = 0$ and $A_i = 0$ we may neglect F_M since when $M_i = 0$ the equation

$$\frac{dS_i}{dt} = P_M F_M (M_o - \lambda$$

[13] This would also be true of the penetration

becomes

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o$$

where $\left(\frac{dS_i}{dt}\right)_b$ is the rate when $M_i = A_i = 0$.

Case II.—Let us now consider the penetration of ions.[14] We assume that the outer surface of the protoplasm consists of a non-aqueous layer through which A^- cannot pass without H^+ so that the ions will enter as ion pairs ($H^+ + A^-$) for which we assume that simultaneous collision with the surface is necessary. Hence the rate of entrance will be proportional to the number of such collisions which is in turn proportional to the product of H_o by A_o. (We omit for the present consideration of exchange of ions of the same sign.)

Just as we consider the permeability of the protoplasm to molecules, P_M, to be equal to the amount of HA entering in unit time through unit surface in molecular form[3] when $M_o - M_i = 1$ so we may consider the permeability of the protoplasm to the ion pair $H^+ + A^-$ to be the amount of HA entering in ionic form ($H^+ + A^-$) in unit time through unit surface when $(H_o)(A_o) - (H_i)(A_i) = 1$ (*i.e.*, when $KM_o - KM_i = 1$). The value of P_A will depend on a "collision" factor G_{H+A} (analogous to G_M) and on a factor D_{H+A} (analogous to D_M) to which we may add a proportionality factor H_{H+A} (analogous to H_M) and write

$$P_A = (H_{H+A})(D_{H+A})(G_{H+A})$$

Letting A_o denote the external and A_i the internal concentration of A^- we may regard the amount entering in unit time at the beginning (when $M_i = 0$ and $A_i = 0$) as $P_A H_o A_o$ which is equal to $P_A K M_o$ (since $H_o A_o = K M_o$).

Hence when $M_i = 0$ and $A_i = 0$ the amount entering in unit time is $P_A K M_o$ and when $M_o = 0$ and $A_o = 0$ the amount passing out in unit time is $P_A K M_i$. When the amounts are expressed as mols it

[14] We shall, for convenience, assume that this is independent of that of molecules. If some ions combine at the surface of the protoplasm to make molecules which pass through the protoplasm in undissociated form and dissociate on the other side it will not affect our calculations except to substitute P_M for P_A.

We neglect for the present any exchange of ions of like sign as this will be taken up later.

is evident that the amount of HA entering in ionic form is equal to the amount of S_i entering so that we may put

$$\frac{dS_i}{dt} = P_A K M_o - P_A K M_i = P_A K (M_o - M_i)$$

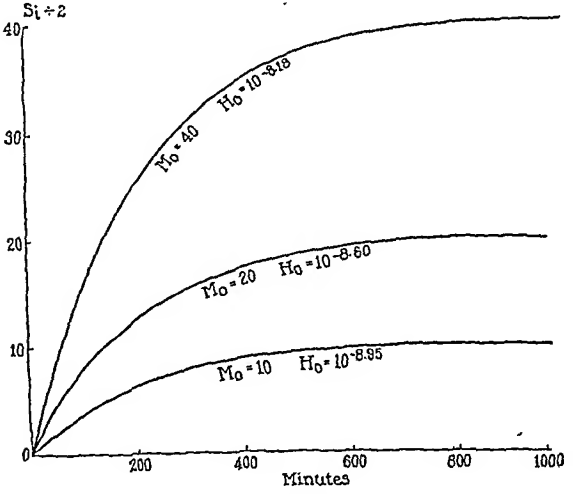


FIG. 3. Calculated time curves of S_i (total amount inside) when ions alone penetrate and F_M and S_o are constant but H_o varies (and in consequence M_o and M_i, vary). It is assumed that $K = 10^{-8}$, $S_o = 100$, $F_M = 0.5$, and $V_A = 0.001$. The equation is $V_A = \frac{1}{t} \ln_e \frac{S_o}{S_o - S_i}$.

The same curves would serve if molecules alone entered by putting $V_M = 0.001$ in place of $V_A = 0.001$ or if both molecules and ions entered by putting $V_{MA} = 0.001$ in place of $V_A = 0.001$. In all these cases the rate rises as the external pH value decreases.

(If F_M remains constant the value of $\frac{dS_i}{dt}$ will increase as the external pH value falls and M_o increases, as in Fig. 3.)

Multiplying the right-hand side by $\frac{F_M}{F_M}$ we have

$$\frac{dS_i}{dt} = P_A K F_M \left(\frac{M_o}{F_M} - \frac{M_i}{F_M}\right) = P_A K F_M (S_{ie} - S_i)$$

Putting $P_A K F_M = V_A$ we have

$$\frac{dS_i}{dt} = V_A (S_{ie} - S_i)$$

and

$$V_A = \frac{1}{t} \ln_o \frac{S_{ie}}{S_{ie} - S_i}$$

Also[15]

$$\frac{dM_i}{dt} = \frac{dS_i}{dt} F_M = V_A (M_o - M_i)$$

---

[15] This may be illustrated as follows. If the time unit is sufficiently short we may regard the rate as uniform during any one interval and at the beginning (when $M_i = A_i = 0$) we may assume that during the first interval of time the egress is negligible. If $M_o = 10{,}000$ millimols per liter, $P_A = 0.5$, $K = 0.001$ and $F_M = 0.8$ the amount leaving the external solution in the first unit of time is $P_A K M_o = 5$ a part of which is at once converted to ions so that only (5)(0.8) = 4 appear as M_i. In the next unit of time a part of this goes out, amounting to $P_A K M_i = 0.002$, leaving $4 - 0.002 = 3.998$ M_i and 1 A_i so that $M_i + A_i = 4.998$, but ions must combine until we have $M_i = F_M(M_i + A_i) = (0.8)(4.998) = 3.9984$ M_i so that the real loss of $M_i = 4 - 3.9984 = 0.0016$ or $P_A K F_M M_i$. The amount of M_i coming in during the second unit of time is, as before, $P_A K F_M M_o = 4$. The net increase of M_i in the second unit of time, which we may call $\frac{\Delta M_i}{dt}$, is the difference between the incoming and the outgoing quantities or $\frac{\Delta M_i}{dt} = P_A K F_M M_o - P_A K F_M M_i = P_A K F_M (M_o - M_i)$ and putting $P_A K F_M = V_A$, the apparent velocity constant of the process, we have

$$\frac{\Delta M_i}{dt} = V_A (M_o - M_i)$$

and for the first two units of time we have

$$V_A = \frac{1}{t} \ln_o \frac{M_o}{M_o - M_i} = \frac{1}{2} \ln_o \frac{10{,}000}{10{,}000 - 7.9984} = 0.0004$$

which agrees with the originally assumed values, *i.e.* $V_A = P_A K F_M = (0.5)(0.001)(0.8) = 0.0004$, whence $P_A = 0.5$ (this value is approximate only; values calculated in this way approach the more closely the assumed value the smaller this value is taken).

and

$$\frac{dA_i}{dt} = \frac{dS_i}{dt} F_A = V_A(S_{ie}F_A - S_{ie}F_A) = V_A(A_{ie} - A_i)$$

and

$$\left(\frac{dS_i}{dt}\right)_b = P_A K M_o$$

That the penetration of ions must be proportional to the ionic product $(H_o)(A_o)$ is evident from the condition at equilibrium where the amount going in is $P_A H_o A_o$ which must be equal to the amount coming out or $P_A H_i A_i$; this is correct if we regard the amount going in as $P_A H_o A_o$ and the amount coming out as $P_A H_i A_i$ since at equilibrium $H_o A_o = H_i A_i$, but it could not be correct where H_i is not equal to H_o (as in the case of a Donnan equilibrium) unless the penetration of ions were proportional to the ionic product. (If molecules alone penetrated the amounts going in and out would be equal despite the inequality of A_o and A_i since M_o would be equal to M_i.)

Case III.—If both molecules and ions go in simultaneously the total amount of S_i passing in (both as ions and as molecules) when $M_i = 0$ and $A_i = 0$ is $P_M M_o + P_A K M_o$. The total amount passing out when $M_o = 0$ and $A_o = 0$ is $P_M M_i + P_A K M_i$. The net amount passing in may be regarded as

$$\frac{dS_i}{dt} = (P_M M_o + P_A K M_o) - (P_M M_i + P_A K M_i) = (P_M + P_A K)(M_o - M_i)$$

When $M_i = A_i = 0$ we may put

$$\left(\frac{dS_i}{dt}\right)_b = (P_M + P_A K) M_o$$

Putting $P_M + P_A K = V$, we have[16]

$$\frac{dS_i}{dt} = V(M_o - M_i) = VF_M\left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right) = VF_M(S_{ie} - S_i)$$

[16] Although the velocity constant is VF_M nevertheless for any given value of M_o and M_i the value of $\frac{dS_i}{dt}$ is independent of F_M because when we double F_M we halve S_{ie} since $S_{ie} = \frac{M_o}{F_M}$.

Putting $VF_M = V_{MA}$ we have

$$\frac{dS_i}{dt} = V_{MA}(S_{ie} - S_i)$$

Also[17]

$$\frac{dM_i}{dt} = F_M \frac{dS_i}{dt} = F_M V(M_o - M_i) = V_{MA}(M_o - M_i)$$

and

$$\frac{dA_i}{dt} = F_A \frac{dS_i}{dt} = V_{MA}(S_{ie}F_A - S_iF_A) = V_{MA}(A_{ie} - A_i)$$

---

[17] This may be illustrated as follows. Let us put $M_o = 10{,}000$, $P_M = 0.01$, $P_A = 0.5$, $K = 0.001$, $F_M = 0.4$, and $F_A = 0.6$ (we need not assign any value to A_o since it does not enter into our calculations). We then have $V = P_M + P_AK = 0.0105$ and $V_{MA} = VF_M = (0.0105)(0.4) = 0.0042$. At the start when $M_i = A_i = 0$ the molecules entering in the first unit of time $= P_MM_o = (0.01)\ (10{,}000) = 100$ and the amount of A^- entering in ionic form is $P_AKM_o = (0.5)\ (0.001)\ (10{,}000) = 5$, the total being $100 + 5 = 105$ of which $(0.4)\ (105) = 42 = M_i$. During the second unit of time a part of this diffuses out: this part amounts to $[P_MM_i = (0.01)\ (42) = 0.42]$ plus $[P_AKM_i = (0.5)\ (0.001)\ (42) = 0.021]$, the total amount escaping being $0.42 + 0.021 = 0.441$ which must be subtracted from the amount entering in the second unit of time (105) to get the net increase in S_i: this net increase is

$$\frac{\Delta S_i}{dt} = 105 - 0.441 = 104.559$$

of which $M_i = (0.4) \cdot 104.559 = 41.8236$. As a check we may calculate this by the formula given above

$$\frac{\Delta S_i}{dt} = V(M_o - M_i)$$

and get $\frac{\Delta S_i}{dt} = (0.0105)\ (10{,}000 - 42) = 104.559$. Also we may put for the first unit of time

$$\frac{\Delta M_i}{dt} = V_{MA}(M_o - M_i) = (0.0042)\ (10{,}000) = 42$$

We may calculate the value of V_{MA} from the equation

$$V_{MA} = \frac{1}{t} \ln_e \frac{M_i}{M_o - M_i}$$

The correctness of these equations may be tested by considering conditions at equilibrium when the amount coming out is known (since it must be equal to the amount going in). The amount entering in unit time when $M_i = 0$ and $A_i = 0$ may be regarded as $P_M M_o + P_A K M_o$. According to the equations given above[18] the amount coming out when $M_o = 0$ and $A_o = 0$ is $P_M M_i + P_A K M_i$. Hence $P_M M_o + P_A K M_o = P_M M_{ie} + P_A K M_{ie}$: this is evidently correct since at equilibrium $M_o = M_{ie}$.

It is evident that the velocity constant when both ions and molecules enter is equal to the velocity constant when molecules alone enter plus the velocity constant when ions alone enter. We have

(1) Molecules alone entering

$$\frac{dS_i}{dt} = P_M F_M (S_{ie} - S_i) = V_M (S_{ie} - S_i)$$

(2) Ions alone entering

$$\frac{dS_i}{dt} = P_A F_M K (S_{ie} - S_i) = V_A (S_{ie} - S_i)$$

(3) Both ions and molecules entering

$$\frac{dS_i}{dt} = (P_M F_M + P_A F_M K)(S_{ie} - S_i) = (V_M + V_A)(S_{ie} - S_i)$$

Evidently $V_M + V_A = (P_M F_M + P_A F_M K)(S_{ie} - S_i) = F_M (P_M + P_A K)(S_{ie} - S_i) = V_{MA}$ so that we have

$$\frac{dS_i}{dt} = V_{MA}(S_{ie} - S_i)$$

For the first unit of time we have

$$V_{MA} = \ln_e \frac{10{,}000}{10{,}000 - 42} = 0.0042$$

and for the first two units of time

$$V_{MA} = \frac{1}{2} \ln_e \frac{10{,}000}{10{,}000 - (42 + 41.8236)} = 0.0042$$

which agrees with the assumed values $V_{MA} = VF_M = (0.4)(0.01 + 0.005) = 0.0042$. These values are approximate only; values calculated in this way approach nearer to the assumed values the smaller they are taken.

[18] When HA is the only solute present A_o is approximately equal to H_o (except at very low concentrations) so that we may put $(H_o)(A_o) = KM_o = (H_o)^2 = (A_o)^2$.

Since all these velocity constants are functions of F_M they depend on the internal pH value: but they are independent of the external pH value (unless this affects the value of F_M).

Even if the external concentration of HA is kept constant so that the initial diffusion gradient is always the same the value of S_{ie} may vary since at equilibrium $M_o = M_{ie}$ but A_{ie} varies with F_M (the smaller F_M the greater is A_{ie}). If in every case the same amount of HA enters in the first unit of time this will form a smaller fraction of S_{ie} when S_{ie} is large and F_M is small and hence the velocity constant must be small when F_M is small. This statement is based on equations involving S_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$, and is equally true of V_M, V_A, and V_{MA}. It also applies to equations involving M_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$, for if F_M is large the amount of HA entering in the first unit of time will remain largely in the form of M_i and consequently constitute a larger fraction of M_{ie} than when F_M is small: so that the velocity constant V_M must increase with F_M (and this applies equally to V_A and V_{MA}). Similar considerations apply to equations involving A_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$.

Although we have $\frac{dS_i}{dt} = P_M F_M (S_{ie} - S_i)$ this value is independent of F_M since when we double F_M we halve S_{ie} and S_i as is evident from the equation $\frac{dS_i}{dt} = P_M F_M \left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right)$. This applies also to $\frac{dS_i}{dt}$ in Cases II and III.

If we keep the total outside concentration ($M_o + A_o$) constant and vary the pH value, by adding a base which penetrates slowly or not at all,[19] while the inside pH value remains constant, a decrease in the external pH value will increase the value of $M_o = M_{ie}$ and of $S_{ie} = M_{ie} + A_{ie}$ (*i.e.*, the total inside concentration at equilibrium) and like-

[19] The actual rate can be calculated by means of the equations given later for the penetration of $Na^+ + A^-$.

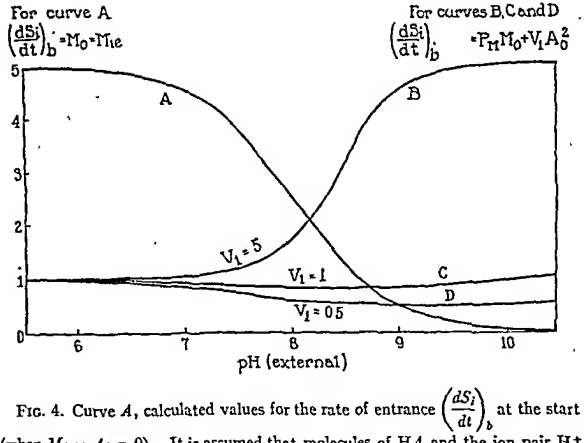


FIG. 4. Curve *A*, calculated values for the rate of entrance $\left(\frac{dS_i}{dt}\right)_b$ at the start (when $M_i = A_i = 0$). It is assumed that molecules of HA and the ion pair $H^+ + A^-$ penetrate, that $K = 10^{-8}$, $S_o = 5$, F_M is constant and that H_o (and in consequence M_o and M_{ie}) varies. The equation is $\left(\frac{dS_i}{dt}\right)_b = (P_M + P_A K)\, M_o$ where $P_M + P_A K = 1$ this becomes $\left(\frac{dS_i}{dt}\right)_b \times 10^8 = M_o = M_{ie}$.

The same curve would serve if molecules alone entered by putting $P_M = 1$ in place of $P_M + P_A K$ or if ions alone entered by putting $P_A K = 1$ in place of $P_M + P_A K$. In all these cases the initial rate $\left(\frac{dS_i}{dt}\right)_b$ falls off in the same manner as the external pH value increases.

Curves *B*, *C*, and *D*, calculated values for the rate of entrance $\left(\frac{dS_i}{dt}\right)_b$ at the start (when $M_i = A_i = 0$). It is assumed that molecules of HA and the ion pair $Na^+ + A^-$ enter, that $S_o = 1$, F_M is constant, $P_M = 1$, that $Na_o = A_o$, and that V_1 varies (*cf.* Fig. 7). The equation is $\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2$ where $V_1 = P_{NaA}$.

When $H_o = H_i$ the velocity constant V_{MNa} behaves somewhat like $\left(\frac{dS_i}{dt}\right)_b$ in Curves *B*, *C*, and *D*: when H_i is constant it behaves like V_{Na} (upper curve) in Fig. 5.

wise the total amount $\left(\frac{dS_i}{dt}\right)$ penetrating in unit time, no matter whether molecules alone penetrate, or ions alone, or both together[20] (Figs. 3 and 4). This will be true even if the inside pH value changes during the process of penetration. Hence it is not possible to decide on this basis whether ions enter.

In case the cell has a slowly diffusible ion in place of an indiffusible ion these remarks will be approximately true[21] but there will be a tendency to reach a temporary pseudoequilibrium after which there will be a drift toward true equilibrium (at which the inside and outside pH values and concentrations will be equal) as the result of the movement of the slowly diffusing ions.

The fact that in the case of *Valonia* H_2S and CO_2 quickly reach a pseudoequilibrium[22] which remains constant indicates that the chief cations of sea water other than H must penetrate slowly or not at all:[23] otherwise A^- would continue to penetrate (paired with some cation other than H^+) until true equilibrium were reached and if this were rapid enough it could be observed experimentally.

Case IV.—If the ion pair $Na^+ + A^-$ alone enters[24] (*i.e.*, the en-

[20] In case both penetrate together the velocity constant will be greater than otherwise, as already shown. In Case III change of H_i may have less effect on the initial rate or the velocity constant.

[21] If Na^+ is inside and comes out very slowly it acts very much like R^+ (in calculations we must bear in mind that it is present on both sides).

[22] It has been shown experimentally for *Valonia* (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–28, 8, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, 9, 255) that if a weak acid penetrates rapidly it soon reaches such a pseudoequilibrium. (In the case of *Valonia* the inside pH value is lower than the outside but this does not affect the principle involved.)

[23] The penetration of CO_2 and H_2S does not prove that H^+ penetrates since they may enter in undissociated form.

[24] The penetration of HA will increase and that of $Na^+ + A^-$ will decrease the value of H_i. As an illustration of what is meant we may assume the following values: at the start $H_o = 10^{-5.51855}$, $M_o = 10^{-3.0371}$, $ROH = 10^{-2.99715}$, $R = 10^{-5.493575}$, $H_i = 10^{-8.501425}$, $A_i = 0$, $M_i = 0$. We assume that the dissociation constant for HA is $K = 10^{-8}$ and for $ROH = K_B = 10^{-8}$. After penetration of HA has occurred (the outside being assumed to remain constant) we have $R_{ie} = 10^{-4.0371}$, $OH_{ie} = 10^{-7}$, $ROH = 10^{-3.0371}$, $H_{ie} = 10^{-7}$, $M_{ie} = 10^{-3.0371}$, $A_{ie} = 10^{-4.0371}$. Let us now add to the outside NaOH and HA until $H_o = 10^{-8}$, A_o

trance of HA in ionic or molecular form is negligible) we may proceed as follows. Just as we put (p. 268) P_A equal to the amount of HA entering in ionic form through unit surface in unit time when $H_oA_o - H_iA_i = 1$ so we may put P_{NaA} equal to the amount of NaA entering in ionic form through unit surface in unit time when $Na_oA_o - Na_iA_i = 1$. The amount of NaA entering in unit time when $M_i = Na_i = A_i = 0$ is $P_{NaA}Na_oA_o$; the amount leaving in unit time when $Na_o = A_o = 0$ is $P_{NaA}Na_iA_i$. Expressing amounts in all cases as mols it is evident that the amount of NaA entering is equal to the amount of S_i entering so that we may write

$$\frac{dS_i}{dt} = P_{NaA}(Na_oA_o - Na_iA_i)$$

(At equilibrium $Na_oA_o = Na_iA_i$.)

Assuming that the ratio of A_i to Na_i is approximately constant during penetration we may put $Na_i = BA_i$ where B is a constant, and for convenience we may put $Na_oA_o = M^2$. We may then write

$$\frac{dS_i}{dt} = P_{NaA}(M^2 - BA_i^2)$$

Assuming that F_A is constant we have (since $A_i = F_AS_i$)

$$\frac{dA_i}{dt} = \frac{dS_i}{dt}F_A = P_{NaA}F_A(M^2 - BA_i^2)$$

On integrating we obtain

$$2P_{NaA}F_AM\sqrt{B} = \frac{1}{t}\ln_e\frac{M + \sqrt{B}A_i}{M - \sqrt{B}A_i}$$

Putting $2P_{NaA}F_AM\sqrt{B} = V_{Na}$ we have

$$V_{Na} = \frac{1}{t}\ln_e\frac{\sqrt{Na_oA_o} + \sqrt{B}A_i}{\sqrt{Na_oA_o} - \sqrt{B}A_i}$$

This gives a curve whose velocity constant, calculated as equal to $\frac{1}{t}\ln_e\frac{A_{ie}}{A_{ie} - A_i}$, increases from the start.[25]

$= 10^{-3}$, $M_o = 10^{-3}$, $Na_o = 10^{-3}$. After penetration we shall have at equilibrium $ROH = 10^{-3}$, $R_{ie} = 10^{-5.002}$, $H_{ie} = 10^{-8.002}$, $A_{ie} = 10^{-2.998}$, $Na_{ie} = 10^{-3.002}$, $M_{ie} = 10^{-3}$ while the outside is assumed to remain unchanged. We see that H_{ie} changes from $10^{-8.5}$ to 10^{-7}.

[25] By way of illustration we may put $A_o = Na_o = 10$, $Na_i = A_i$, $F_A = 0.5$,

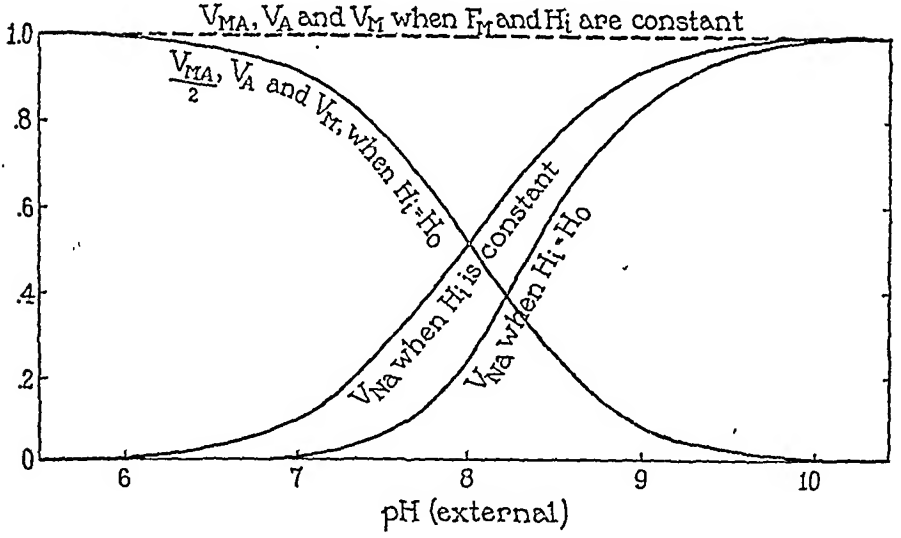


FIG. 5. Shows the change of V_M, V_A, and V_{MA} with change of external pH value (on the supposition that at pH 5.5 $V_M = V_A = V_{MA} = 1$) and also the change of V_{Na} (assuming that its value at pH 10.5 is 1). It is assumed that $S_o = 1$, $K = 10^{-8}$ and $Na_o = A_o$.

As long as F_M remains constant V_M, V_A, and V_{MA} remain constant but if R^+ is so small that H_i is approximately equal to H_o these velocity constants will fall off as the external pH value increases. The equations are $V_M = P_M F_M$; $V_A = P_A K F_M$; $V_{MA} = (P_M + P_A K) F_M$; $V_{Na} = 2P_{NaA} F_A \sqrt{Na_o A_o} \sqrt{B}$.

When H_i is not equal to H_o we put $P_M = P_A K = F_M = 1$ (for V_{MA} we put $F_M = 0.5$) so that $V_M = V_A = V_{MA} = 1$ and when $2P_{NaA} F_A \sqrt{B} = 1$ we have $V_{Na} = A_o$.

When $H_i = H_o$ we have $F_M = M_o$ and may put $V_M = V_A = V_{MA} \div 2 = M_o$. Putting $2P_{NaA} \sqrt{B} = 1$ we have (since $F_A = A_o$) $V_{Na} = A_o^2$.

In general the behavior of V_{MNa} when H_i is constant is like that of V_{Na} when H_i is constant. When $H_i = H_o$ V_{MNa} behaves like $\left(\frac{dSi}{dt}\right)_b$ in Curves *B*, C, and *D* in Fig. 4.

$B = 1$, and $P_{NaA} = 0.01$. Then $V_{Na} = 2P_{NaA} F_A A_o \sqrt{B} = 0.1$. In the first unit of time the total amount S_i entering (when $A_i = Na_i = 0$) is

$$\frac{\Delta S_i}{dt} = P_{NaA} A_o^2 = (0.01)\ 10^2 = 1$$

of which half is A_i since $F_A = 0.5$ so that $A_i = 0.5$ (we assume that none goes out during the first unit of time). In the second unit of time the amount of S_i entering is 1 and the amount going out is $P_{NaA} A_i^2 = 0.01(0.5)^2 = 0.0025$ so that the

We may also write

$$V_{N_a} = \frac{1}{t}\ln_e \frac{(\sqrt{Na_oA_o} + \sqrt{BA_i}) + F_A}{(\sqrt{Na_oA_o} - \sqrt{BA_i}) + F_A} = \frac{1}{t}\ln_e \frac{(\sqrt{Na_oA_o} + F_A) + \sqrt{BS_i}}{(\sqrt{Na_oA_o} + F_A) - \sqrt{BS_i}}$$

Let us now consider what happens when the total external concentration S_o($= M_o + A_o$) is kept constant and the pH value is varied. Let us suppose that at the start HA alone is present and that we add increasing amounts of NaOH. We then have $V_{N_a} = 2P_{N_aA}F_A\sqrt{Na_oA_o}\sqrt{B}$ and if $Na_o = A_o$ we have $V_{N_a} = 2P_{N_aA}F_AA_o\sqrt{B}$ so that if F_A is constant V_{N_a} is directly proportional to A_o, which increases with increase of external pH value: if R^+ is small so that we can write as an approximation $H_o = H_i$ and if we also put $S_o = 1$ we have $F_A = A_o$. Putting $2P_{N_aA}\sqrt{B} = 1$ we have $V_{N_a} = F_AA_o = A_o^2$ (see Fig. 5). The initial rate $\left(\frac{dS_i}{dt}\right)_b$ likewise increases with the external pH value since we have

$$\left(\frac{dS_i}{dt}\right)_b = P_{N_aA}Na_oA_o$$

and putting $P_{N_aA} = V_1$ this becomes

$$\left(\frac{dS_i}{dt}\right)_b = V_1Na_oA_o$$

Then

$$\left(\frac{dA_i}{dt}\right)_b = F_A\left(\frac{dS_i}{dt}\right)_b = V_1F_ANa_oA_o$$

Putting $V_1F_A = V_2$ we have

$$\left(\frac{dA_i}{dt}\right)_b = V_2Na_oA_o$$

---

net increase in S_i is $1 - 0.0025 = 0.9975$ of which half or 0.49875 is A_i. Hence at the end of the second period $A_i = 0.5 + 0.49875 = 0.99875$ and

$$V_{N_a} = \frac{1}{t}\ln_e \frac{10 + 0.99875}{10 - 0.99875} = 0.1$$

This is approximate (in all such cases the approximation is nearer the smaller the value of V_{N_a}).

Case V.—Molecules of HA *and the Ion Pair* $Na^+ + A^-$ *Enter.* We then have

$$\frac{dA_i}{dt} = V_M(A_{ic} - A_i) + V_2(Na_oA_o - Na_iA_i)$$

If the proportion of Na_i to A_i remains sufficiently constant during penetration we may write as an approximation $Na_i = BA_i$. Putting $V_MA_{ic} + V_2Na_oA_o = E$ we have

$$\frac{dA_i}{dt} = E - V_MA_i - V_2BA_i^2$$

On integrating (between 0 and A_{ic}) we obtain

$$\sqrt{V_M^2 + 4EV_2B} = \frac{1}{t}\ln_e \frac{\sqrt{V_M^2 + 4EV_2B} + 2V_2BA_i + V_M}{\sqrt{V_M^2 + 4EV_2B} - 2V_2BA_i - V_M}\left(\frac{\sqrt{V_M^2 + 4EV_2B} - V_M}{\sqrt{V_M^2 + 4EV_2B} + V_M}\right)$$

Putting $\sqrt{V_M^2 + 4EV_2B} = V_{MNa}$ and $2V_2B = V_3$ we have

$$V_{MNa} = \frac{1}{t}\ln_e \frac{V_{MNa} + V_3A_i + V_M}{V_{MNa} - V_3A_i - V_M}\left(\frac{V_{MNa} - V_M}{V_{MNa} + V_M}\right)$$

Putting $V_{MNa} + V_M = V_4$ and $V_{MNa} - V_M = V_5$ we have[26]

$$V_{MNa} = \frac{1}{t}\ln_e \frac{V_4 + V_3A_i}{V_5 - V_3A_i}\left(\frac{V_5}{V_4}\right)$$

---

[26] As an illustration we may put $M_o = M_{ic} = 22.2$, $A_o = Na_o = 10$, $A_{ic} = 11.1$, $F_M = 0.667$, $F_A = 0.333$, $B = 0.811$, $P_M = 0.0015$, $P_{NaA} = 0.003$, $V_M = P_MF_M = 0.001$, and $V_2 = P_{NaA}F_A = 0.001$. In the first unit of time the amount going in is

$$\frac{\Delta S_i}{dt} = P_MM_o + P_{NaA}Na_oA_o = (0.0015)22.2 + (0.003)(10)(10) = 0.3333$$

We then have $A_i = F_AS_i = (0.333)(0.3333) = 0.1111$ and $M_i = F_MS_i = 0.2222$. We may assume for convenience that none of this goes out during the first unit of time but that during the second unit of time the amount going out is $P_MM_i + P_{NaA}Na_iA_i = P_MM_i + P_{NaA}BA_i^2 = (0.0015)(0.222) + (0.003)(0.811)(0.1111)^2 = 0.00036291$. This must be subtracted from the total amount going in during the first two units of time (which is $2(0.3333) = 0.6666$) to get the net total inside at the end of the second unit of time: this is $0.6666 - 0.00036291 = 0.666237$ of which 1/3 or 0.222079 is A_i. We may arrive at the same result by using the formula

$$\frac{\Delta A_i}{dt} = V_M(A_{ic} - A_i) + V_2(Na_oA_o - BA_i^2)$$

This gives a curve whose velocity constant calculated as equal to $\frac{1}{t}\ln_e \frac{A_{ie}}{A_{ie} - A_i}$ increases from the start.

Let us now consider the significance of V_{MNa}. We have $V_{MNa} = \sqrt{V_M^2 + 4EV_2B}$. Substituting the value of E and putting $Na_o = A_o$ we have

$$V_{MNa} = \sqrt{V_M^2 + 4V_MV_2BA_{ie} + 4V_2^2BA_o^2}$$

If R^+ is small we may write as an approximation $B = 1$ and $A_o = A_{ie}$. We then have

$$\begin{aligned} V_{MNa} &= \sqrt{V_M^2 + 4V_MV_2A_o + 4V_2^2A_o^2} \\ &= V_M + 2V_2A_o \\ &= P_MF_M + 2P_{Na}AF_AA_o \end{aligned}$$

so that in case F_M and F_A are approximately constant V_{MNa} will increase as A_o increases, *i.e.*, with increase of external pH value.[27]

---

and putting $A_i = 0$ at the start. We then have for the first unit of time

$$\frac{\Delta A_i}{dt} = V_MA_{ie} + V_2Na_oA_o$$

or $(0.001)(11.1) + (0.001)(10)(10) = 0.1111$. At the end of the first unit of time we have $A_i = 0.1111$ and for the second unit of time

$$\frac{\Delta A_i}{dt} = V_M(A_{ie} - A_i) + V_2(Na_oA_o - BA_i) = (0.001)(11.1 - 0.1111) +$$

$$(0.001)\,[100 - (0.811)\,(0.1111)^2] = 0.110979$$

adding this to the amount of A_i present at the beginning of the second unit of time we have $0.1111 + 0.110979 = 0.222079\ A_i$.

For the first unit of time we have

$$t = \frac{1}{V_{MNa}}\ln_e \frac{V_4 + V_3A_i}{V_3 - V_3A_i}\left(\frac{V_3}{V_4}\right) = \frac{1}{0.019}\ln_e \frac{0.020 + 0.001622\,(0.1111)}{0.018 - 0.001622\,(0.1111)}\left(\frac{0.018}{0.020}\right) = 1$$

For the first two units of time taken together we have

$$t = \frac{1}{0.019}\ln_e \frac{0.020 + 0.001622\,(0.222079)}{0.018 - 0.001622\,(0.222079)}\left(\frac{0.018}{0.020}\right) = 2$$

These values are approximate: they approach nearer to the assumed values of t the smaller the values assumed for V_M and V_2.

[27] This would also be true if we did not assume that $A_o = A_{ie}$ or that $Na_o = A_o$ since we should have $V_{MNa} = \sqrt{\text{constant} + Na_oA_o}$.

But if the internal pH value rises with the external F_M will fall off. If R^+ is small we may write as an approximation $H_o = H_{ic}$. If H_i does not change greatly during penetration and if $S_o = M_o + A_o = 1$ we have $F_M = M_o$ and $F_A = A_o$. We then have $V_{MNa} = P_M M_o + P_{NaA} A_o^2$ so that the behavior of V_{MNa} will resemble that of $\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2 = P_M M_o + P_{NaA} A_o^2$ in curves B, C, and D in Fig. 4. Hence if the value of P_{NaA} is small in comparison with that of P_M we shall expect V_{MNa} to fall off as the external pH value rises but if the value of P_{NaA} is large in comparison with that of P_M we shall expect V_{MNa} to increase as the external pH value increases.

We also have

$$\frac{dS_i}{dt} = \frac{dA_i}{dt}\left(\frac{1}{F_A}\right) = (E - V_M A_i - V_2 B A_i^2) \div F_A$$

and

$$V_{MNa} = \frac{1}{t} \ln_e \frac{V_4 \div F_A + V_3 S_i}{V_5 \div F_A - V_3 S_i}\left(\frac{V_5}{V_4}\right)$$

and

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 Na_o A_o$$

Let us now consider the behavior of $\left(\frac{dS_i}{dt}\right)_b$ with increase in external pH value. In the beginning when $M_i = A_i = 0$ the amount going in as molecules in unit time is $P_M M_o$ and if $Na_o = A_o$ the amount of S_i entering in ionic form is $V_1 Na_o A_o = V_1 A_o^2$. If we put $P_M = V_1 = 1$, keep the total external concentration ($M_o + A_o = S_o$) constant, and change the dissociation of HA by adding various quantities of NaOH we may calculate the total amount entering, *i.e.*,

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2 = M_o + A_o^2$$

for each value of M_o.[28] Fig. 6 shows the results of such calculations

[28] *E.g.*, if we put $P_M = V_2 = S_o = 1$, $M_o = 0.5$ (*i.e.*, $M_o = 50$ per cent), and $A_o = 0.5$, we have

$$\left(\frac{\Delta S_i}{dt}\right)_b = 0.5 + (0.5)^2 = 0.75$$

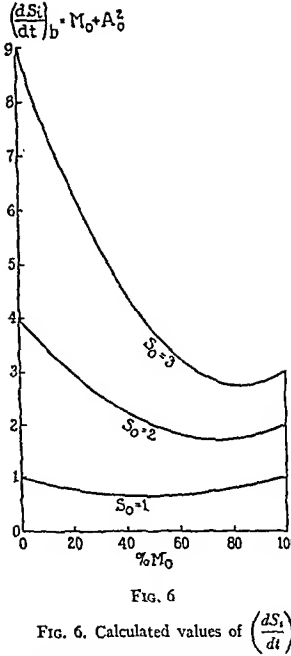


FIG. 6

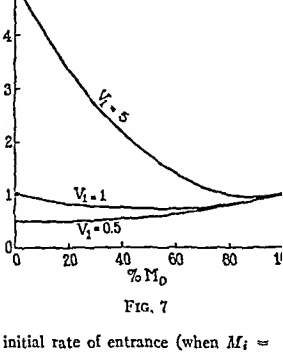


FIG. 7

FIG. 6. Calculated values of $\left(\frac{dS_i}{dt}\right)_b$ = initial rate of entrance (when $M_i = A_i = 0$) if molecules of HA and the ion pair $Na^+ + A^-$ enter. The equation is

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + P_{NaA} Na_o A_o$$

We assume that $Na_o = A_o$ and that $P_M = P_{NaA} = 1$. We then have

$$\left(\frac{dS_i}{dt}\right)_b = M_o + A_o^2$$

FIG. 7. Calculated values of $\left(\frac{dS_o}{dt}\right)_b$ = initial rate of entrance (when $M_i + A_i = 0$) if molecules of HA and the ion pair $Na^+ + A^-$ enter. The equation is

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 Na_o A_o$$

where $V_1 = P_{NaA}$. We assume that $Na_o = A_o$, $P_M = S_o = 1$, but V_1 varies.

for $S_o = 1$, $S_o = 2$, and $S_o = 3$. It is evident that $\left(\frac{dS_i}{dt}\right)_b$ passes through a minimum which is characteristic for each value of S_o. If we put $S_o = P_M = 1$ and assume various values for V_1 we see that above a certain value the curves (as shown in Figs. 4 and 7) pass through a minimum.

Case VI.—If ions alone enter we may consider as an approximation that the ion pairs $H^+ + A^-$ and $Na^+ + A^-$ enter independently. The entrance of A will be proportional to the number of collisions and hence to $(H + Na)A = (H)(A) + (Na)(A)$: it will also depend on the permeability which may differ for $Na + A$ and $H + A$, hence it would seem to be better to put the number of collisions proportional to $(H)(A) + (Na)(A)$ and treat the penetration of $H^+ + A^-$ and $Na^+ + A^-$ as if they were independent. In that case we have (see pp. 271 and 279)

$$\frac{dA_i}{dt} = V_A(A_{ic} - A_i) + V_2(Na_oA_o - Na_iA_i)$$

If we may put $Na_i = BA_i$ and $V_AA_{ic} + V_2Na_oA_o = E_1$ we may write

$$\frac{dA_i}{dt} = E_1 - V_AA_i - V_2BA_i^2$$

On integrating (between 0 and A_{ic}) and putting $\sqrt{V_A^2 + 4E_1V_2B} = V_{ANa}$ and $2V_2B = V_3$ we have

$$V_{ANa} = \frac{1}{t}\ln_e\frac{V_{ANa} + V_3A_i + V_A}{V_{ANa} - V_3A_i - V_A}\left(\frac{V_{ANa} - V_A}{V_{ANa} + V_A}\right)$$

We also have

$$\frac{dS_i}{dt} = \frac{dA_i}{dt}\left(\frac{1}{F_A}\right) = (E_1 - V_AA_i - V_2BA_i^2) \div F_A$$

and

$$V_{ANa} = \frac{1}{t}\ln_e\frac{(V_{ANa} + V_A) \div F_A + V_3S_i}{(V_{ANa} - V_A) \div F_A - V_3S_i}\left(\frac{V_{ANa} - V_A}{V_{ANa} + V_A}\right)$$

$$\left(\frac{dS_i}{dt}\right)_b = P_AH_oA_o + V_1Na_oA_o = P_AKM_o + V_1Na_oA_o$$

It is evident that if S_o remains constant and the external pH value increases H_oA_o will decrease (since $H_oA_o = KM_o$) but Na_oA_o will

increase. The value of $\left(\frac{dS_i}{dt}\right)_b$ will pass through a minimum as in Case V.

In case we add a neutral salt, *e.g.*, LiCl, the number of collisions is proportional to (H + Na + Li) (A + Cl) = (H + Na + Li)(A) + (H + Na + Li)(Cl). Hence the addition of a neutral salt whose cation enters freely should favor the penetration of A^- providing it does not diminish protoplasmic permeability or otherwise inhibit the process.

Case VII.—If molecules of HA together with the ion pairs $H^+ + A^-$ and $Na^+ + A^-$ enter we have

$$\frac{dA_i}{dt} = V_{MA}(A_{it} - A_i) + V_2(Na_o A_o - Na_i A_i)$$

Putting $E_2 = V_{MA}A_{it} + V_2 Na_o A_o$ and $Na_i = BA_i$ we have

$$\frac{dA_i}{dt} = E_2 - V_{MA}A_i - V_2 B A_i^2$$

Putting $\sqrt{V_{MA}^2 + 4E_2V_2B} = V_{MAN_a}$ and $2V_2B = V_3$ we have

$$V_{MAN_a} = \frac{1}{t}\ln_e \frac{V_{MAN_a} + V_3A_i + V_{MA}}{V_{MAN_a} - V_3A_i - V_{MA}} \left(\frac{V_{MAN_a} - V_{MA}}{V_{MAN_a} + V_{MA}}\right)$$

We also have

$$\frac{dS_i}{dt} = (E_2 - V_{MA}A_i - V_2 B A_i^2) + F_A$$

and

$$V_{MAN_a} = \frac{1}{t}\ln_e \frac{(V_{MAN_a} + V_{MA}) + F_A + V_3S_i}{(V_{MAN_a} - V_{MA}) + F_A - V_3S_i} \left(\frac{V_{MAN_a} - V_{MA}}{V_{MAN_a} + V_{MA}}\right)$$

also $$\left(\frac{dS_i}{dt}\right)_b = (P_M + P_A K)M_o + V_1 Na_o A_o$$

The value of $\left(\frac{dS_i}{dt}\right)_b$ will pass through a minimum as in Case V.

Case VIII.—If a weak acid HA and a weak base ZOH enter simultaneously, forming the salt ZA inside, we may use for HA and for ZOH the equations already given (which enable us in some cases to find the amount of HA, H^+, A^-, ZOH, and Z^+ inside at any given moment).

If the dissociation constant, K, of HA is equal to the dissociation constant, K_Z, of ZOH and if HA and ZOH enter in equal amounts the internal pH value will remain almost constant and in consequence F_M and F_{MZ} will remain almost constant (F_{MZ}, the fractional concentration of molecules of ZOH inside $= \frac{M_{Zi}}{M_{Zi} + Z_i}$, where M_{Zi} is the concentration of molecules of ZOH inside and Z_i is the concentration of Z^+ inside).

It is evident from what has been said that when a weak electrolyte such as HA is the only solute penetrating we cannot decide whether ions enter by comparing the rates or the velocity constants at high and low pH values for the relative rates will change in the same way in both cases (*i.e.*, whether molecules alone or ions alone penetrate) and the velocity constants, although differing in the two cases, will act alike. If on the other hand the molecules of a weak electrolyte, HA, and the ions of a salt NaA penetrate together we shall get for $\left(\frac{dS_i}{dt}\right)_b$ a curve like Curve A in Fig. 4 if molecules alone (or ions alone) of HA penetrate, but if the ion pair $Na^+ + A^-$ penetrates in addition to molecules of HA we may expect the types shown in Curves B, C, and D in Fig. 4, as well as those shown in Figs. 6 and 7. The velocity constants will also differ as shown in Fig. 5. If molecules alone (or ions alone) of HA penetrate the velocity constant will remain constant if H_i remains constant, but if H_i approximates H_o the velocity constant will fall off as the external pH value rises; on the other hand the velocity constant will increase with external pH value when the ion pair $Na^+ + A^-$ alone enters. If both molecules of HA and the ion pair $Na^+ + A^-$ enter the velocity constant V_{MNa} may increase or decrease as the case may be. When F_M is constant V_{MNa} acts much like V_{Na} in Fig. 5 (upper curve) but when $H_o = H_i$ it acts much like $\left(\frac{dS_i}{dt}\right)_b$ in Curves B, C, and D in Fig. 4.

In case of an exchange of ions[29] of the same sign going in opposite directions, *e. g.*, exchange of H^+ for Na^+, the total quantity of cations,

[29] Doubtless the cell can produce sufficient ions (*e. g.*, $H^+ + HCO_3^-$) to ensure adequate exchange.

Q, passing inward through the protoplasm in unit time is proportional to the total concentration (H + Na) of cations inside multiplied by that outside so that we may put $Q = L(H_i + Na_i)(H_o + Na_o)$ where L is a proportionality factor and the subscripts i and o denote the concentrations inside and outside respectively (Q will depend on the rate of passage of the slower cation). The quantity of H^+ passing out is equal to $\frac{Q H_i}{H_i + Na_i}$ and that passing in is equal to $\frac{Q H_o}{H_o + Na_o}$ and the net amount passing in $\left(\frac{dH}{dt}\right)$ is the difference between these expressions: on reducing them to a common denominator we get

$$\frac{dH}{dt} = \frac{Q}{(H_o + Na_o)(H_i + Na_i)}(H_oNa_i - H_iNa_o)$$

Now $H_o + Na_o = H_{ob}$ (the concentration of H_o at the start) and $H_i + Na_i = Na_{ib}$ (the concentration of Na_i at the start). Putting $\frac{Q}{H_{ob}Na_{ib}} = U$ we have

$$\frac{dH}{dt} = U(H_oNa_i - H_iNa_o)$$

It is, however, more convenient to proceed as follows. We put

$$\frac{dH}{dt} = \frac{QH_o}{H_o + Na_o} - \frac{QH_i}{H_i + Na_i}$$

$$= \frac{QH_o}{H_{ob}} - \frac{QH_i}{Na_{ib}}$$

$$= U(H_oNa_{ib} - H_iH_{ob})$$

$$= UNa_{ib}\left(\frac{H_oNa_{ib}}{Na_{ib}} - \frac{H_iH_{ob}}{Na_{ib}}\right) = UNa_{ib}\left(H_o - \frac{H_{ob}}{Na_{ib}}H_i\right)$$

Putting $UNa_{ib} = U_1$ and $\frac{H_{ob}}{Na_{ib}} = U_2$ we have

$$\frac{dH}{dt} = U_1(H_o - U_2H_i)$$

If the external solution is relatively large or the concentration of H_o relatively large, so that we may regard H_o as constant, we may put

$$U_1 = \frac{1}{tU_2} \ln_e \frac{H_o}{H_o - U_2 H_i}$$

Experimental tests of these equations are in progress. It may be noted that if we regard the free base of the basic dye brilliant cresyl blue as undissociated we should expect the velocity constant V_M of diffusion into a living cell of *Nitella* to be constant (*cf.* Fig. 5) as long as the pH value of the sap is constant despite the fact that the external pH value changes. This is the result actually found by Irwin.[30]

Let us now consider briefly certain complications due to the fact that the penetrating substance may have different activities in the protoplasm and the aqueous solutions. Overton states that permeability depends largely on the partition coefficient between the outer surface of the protoplasm and the external solution and according to Irwin[31] the partition coefficient at the internal surface is also important.

The equation $\frac{dM_i}{dt} = V_M(M_o - M_i)$ assumes that the partition coefficient between the protoplasm and the external and internal solutions is 1. In case it differs from 1 and is the same for both surfaces of the protoplasm we may call it K_o. If we follow the treatment of Northrop[32] we may put $\frac{dM_i}{dt} = V_M(K_oM_o - K_oM_i) = V_MK_o(M_o - M_i)$. In case it is K_o for the external and K_i for the internal surface[33] (due to a difference between the external solution and the sap) we may put $\frac{dM_i}{dt} = V_M(K_oM_o - K_iM_i)$,[1] whence

$$\frac{dM_i}{dt} = V_MK_o\left(\frac{K_oM_o}{K_o} - \frac{K_iM_i}{K_o}\right).$$

[30] Irwin, M., *J. Gen. Physiol.*, 1922–23, 5, 727.

[31] Irwin, M., *J. Gen. Physiol.*, 1928–29, 12, 407.

[32] Northrop, J. H., *J. Gen. Physiol.*, 1928–29, 12, 435.

[33] For convenience we here regard the protoplasm as a homogeneous non-aqueous phase separating the external solution from the sap.

Putting $\frac{K_i}{K_o} = K_{io}$ and $V_M K_o = V_{M_o}$ we have

$$\frac{dM_i}{dt} = V_{M_o}(M_o - K_{io}M_i)$$

or

$$V_{M_o} = \frac{1}{tK_{io}} \ln_e \frac{M_o}{M_o - K_{io}M_i}$$

For Case II we may proceed as follows. The concentration of molecules in the outer surface of the protoplasm may be called M_{po} and that in the inner surface M_{pi}. If the dissociation constant is K_p we have $H_{po}A_{po} = K_p M_{po}$ and $H_{pi}A_{pi} = K_p M_{pi}$, where the subscripts *po* and *pi* signify concentrations in the outer and inner surfaces respectively. The diffusion gradient for the ion pair $H^+ + A^-$ will therefore be proportional to $H_{po}A_{po} - H_{pi}A_{pi}$ or to $K_p M_{po} - K_p M_{pi}$ which is equal to $K_p K_o M_o - K_p K_i M_i$. In place of the equation already given for Case II, *i.e.* $\frac{dM_i}{dt} = V_A(M_o - M_i)$ we then have

$$\frac{dM_i}{dt} = V_A\,(K_p K_o M_o - K_p K_i M_i)$$

Putting $K_p K_o = K'_o$ and $K_p K_i = K'_i$ we have

$$\frac{dM_i}{dt} = V_A(K'_o M_o - K'_i M_i)$$

and proceeding as before we obtain

$$\frac{dM_i}{dt} = V_A K'_o (M_o - K'_{io} M_i)$$

where $K'_{io} = \frac{K'_i}{K'_o}$. Putting $V_A K'_o = V'_A$ we have

$$V'_A = \frac{1}{tK'_{io}} \ln_e \frac{M_o}{M_o - K'_{io}M_i}.$$

Case III is simply the combination of the equations and presents no difficulty.

For Case IV we may proceed as in Case II. Assuming for purposes of calculation a few undissociated molecules of NaA we may call the dissociation constant of NaA in the protoplasm K'_p. The diffusion gradient is then proportional to $\mathrm{Na}_{po}A_{po} - \mathrm{Na}_{pi}A_{pi} = K'_pK''_oM_o - K'_pK''_iM_i$ where K''_o and K''_i are the partition coefficients for NaA at the outer and inner surfaces and M'_o and M'_i are the concentrations of undissociated molecules of NaA. In place of the equation already given for Case IV, *i.e.* $\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(\mathrm{Na}_oA_o - \mathrm{Na}_iA_i)$ we have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(\mathrm{Na}_{po}A_{po} - \mathrm{Na}_{pi}A_{pi})$$

The relation between $(\mathrm{Na}_{po}A_{po})$ and (Na_oA_o) is obtained as follows

$$K''_o = \frac{M'_{po}}{M'_o} = \frac{\mathrm{Na}_{po}A_{po} \div K'_p}{\mathrm{Na}_oA_o \div K_e}$$

where K_e is the dissociation constant of NaA in the external solution. From this we obtain $\frac{\mathrm{Na}_{po}A_{po}}{\mathrm{Na}_oA_o} = \frac{K''_oK'_p}{K_e}$. We may put $\frac{K''_oK'_p}{K_e} = K'''_o$ and call the corresponding constant for the inner surface K'''_i. We then have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(K'''_o\mathrm{Na}_oA_o - K'''_i\mathrm{Na}_iA_i)$$

$$= P_{\mathrm{Na}A}F_AK'''_o(\mathrm{Na}_oA_o - K'''_{io}\mathrm{Na}_iA_i)$$

where $K'''_{io} = \frac{K'''_i}{K'''_o}$. Putting $M^2 = \mathrm{Na}A_o$, $\mathrm{Na}_i = BA_i$, and $K'''_{io}B = B'$ we have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_AK'''_o(M^2 - B'A_i^2)$$

or

$$2P_{\mathrm{Na}A}F_AM\sqrt{B'} = \frac{1}{t}\ln_e\frac{M + \sqrt{B'}A_i}{M - \sqrt{B'}A_i}$$

Putting $2P_{Na}F_AM\sqrt{B'} = V'_{Na}$ we have

$$V_{Na} = \frac{1}{t}\ln_e \frac{\sqrt{Na_0A_0} + \sqrt{B'}A_t}{\sqrt{Na_0A_0} - \sqrt{B'}A_t}$$

Cases V, VI, VII, and VIII are merely combinations of the equations already considered and hence present no difficulty; they involve only a change of constants.

Hitherto we have treated only those cases where the time curve is of the first order but in practice we may find when we calculate according to the equation $K = \frac{1}{t}\ln_e \frac{a}{a-x}$ that the value of K falls off with time. This might suggest that the curve follows a dimolecular equation but in that case we should expect that on taking any two curves, which we may call I and II, and multiplying all the ordinates of Curve I by a factor to make the final (equilibrium) value equal that of Curve II the two curves would not coincide. For if they do coincide it means that their ordinates at any given time t must bear the same relation as their final equilibrium values. If we are dealing with irreversible reactions the final or equilibrium value of the amount transformed, which we may call x_e, is equal to the amount present at the start, which we may call a. If a monomolecular process giving Curve I M is changed to a monomolecular process giving Curve II M, by simply doubling the amount of a the velocity constant is not changed and the time Curve II M can be reduced to Curve I M by dividing all its ordinates by 2. But if we have a dimolecular process giving Curve I D with the equation $\frac{dx}{dt} = K(a-x)^2$ or $K = \frac{1}{t}\frac{x}{a(a-x)}$ we cannot double the amount of a without changing the velocity constant and Curve II D cannot be reduced to I D by dividing its ordinates by 2. Hence when we have a set of curves with different values of a which are convertible into each other simply by multiplying ordinates we may conclude that they are not of higher orders than the first. Such curves are like those of a reaction of the first order in which the velocity constant diminishes from the start and may for con-

venience be called "inhibited curves." They may often be fitte by empirical formulae such as

$$K = \frac{1}{t} \ln_e \frac{a}{a - bx^n}$$

or

$$K = \frac{ba}{tx^n} \ln_e \left(\frac{a}{a - x}\right) - \frac{b}{x^n}$$

or

$$K = \frac{a}{b^2 t} \ln_e \frac{a}{a - bx} - \frac{x}{b}$$

Similar considerations apply to the exit of electrolytes but in practice there may be complications, such, for example, as those discussed by Irwin.[34]

What is here said of weak acids holds, with suitable modifications, for weak bases and for amphoteric electrolytes, and it may also be applied to strong electrolytes since it is always permissible to assume the existence of a few undissociated molecules for purposes of calculation.

SUMMARY

When the only solute present is a weak acid, HA, which penetrates as molecules only into a living cell according to a curve of the first order and eventually reaches a true equilibrium we may regard the rate of increase of molecules inside as

$$\frac{dM_i}{dt} = P_M F_M (M_o - M_i)$$

where P_M is the permeability of the protoplasm to molecules, M_o denotes the external and M_i the internal concentration of molecules, A_i denotes the internal concentration of the anion A^- and $F_M = \frac{M_i}{M_i + A_i}$. (It is assumed that the activity coefficients equal 1.) Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$\frac{dM_i}{dt} = V_M (M_o - M_i) = V_M (M_{ie} - M_i)$$

[34] Irwin, M., *J. Gen. Physiol.*, 1926–27, 10, 75.

where e denotes the concentration at equilibrium. Then

$$V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$$

where t is time.

The corresponding equation when ions alone enter is

$$\frac{dA_i}{dt} = P_A K F_M (A_{ie} - A_i)$$

where K is the dissociation constant of HA, P_A is the permeability of the protoplasm to the ion pair $H^+ + A^-$, and A_{ie} denotes the internal concentration of A_i at equilibrium. Putting $P_A K F_M = V_A$, the apparent velocity constant of the process, we have

$$\frac{dA_i}{dt} = V_A (A_{ie} - A_i)$$

and

$$V_A = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$$

When both ions and molecules of HA enter together we have

$$\frac{dS_i}{dt} = (V_M + V_A)(S_{ie} - S_i) = V_{MA}(S_{ie} - S_i)$$

where $S_i = M_i + A_i$ and S_{ie} is the value of S_i at equilibrium. Then

$$V_{MA} = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$$

V_M, V_A, and V_{MA} depend on F_M and hence on the internal pH value but are independent of the external pH value except as it affects the internal pH value.

When the ion pair $Na^+ + A^-$ penetrates and $Na_i = BA_i$ we have

$$\frac{dA_i}{dt} = P_{NaA} F_A (Na_o A_o - BA_i^2)$$

and

$$V_{Na} = 2P_{NaA} F_A \sqrt{Na_o A_o B} = \frac{1}{t} \ln_e \frac{\sqrt{Na_o A_o} + \sqrt{B} A_i}{\sqrt{Na_o A_o} - \sqrt{B} A_i}$$

where P_{NaA} is the permeability of the protoplasm to the ion pair $Na^+ + A^-$, Na_o and Na_i are the external and internal concentra-

tions of Na^+, $F_A = \frac{A_i}{S_i}$, $B = \frac{Na_i}{A_i}$, and V_{Na} is the apparent velocity constant of the process.

Equations are also given for the penetration of:

(1) molecules of HA and the ion pair $Na^+ + A^-$,

(2) the ion pairs $H^+ + A^-$ and $Na^+ + A^-$,

(3) molecules of HA and the ion pairs $Na^+ + A^-$ and $H^+ + A^-$.

(4) The penetration of molecules of HA together with those of a weak base ZOH.

(5) Exchange of ions of the same sign.

When a weak electrolyte HA is the only solute present we cannot decide whether molecules alone or molecules and ions enter by comparing the velocity constants at different pH values, since in both cases they will behave alike, remaining constant if F_M is constant and falling off with increase of external pH value if F_M falls off. But if a salt (*e.g.*, NaA) is the only substance penetrating the velocity constant will increase with increase of external pH value: if molecules of HA and the ions of a salt NaA penetrate together the velocity constant may increase or decrease while the internal pH value rises.

The initial rate $\left(\frac{dS_i}{dt}\right)_b$ (*i.e.*, the rate when $M_i = 0$ and $A_i = 0$) falls off with increase of external pH value if HA alone is present and penetrates as molecules or as ions (or in both forms). But if a salt (*e.g.*, NaA) penetrates the initial rate may in some cases decrease and then increase as the external pH value increases.

At equilibrium the value of M_i equals that of M_o (no matter whether molecules alone penetrate, or ions alone, or both together). If the total external concentration ($S_o = M_o + A_o$) be kept constant a decrease in the external pH value will increase the value of M_o and make a corresponding increase in the rate of entrance and in the value at equilibrium no matter whether molecules alone penetrate, or ions alone, or both together.

What is here said of weak acids holds with suitable modifications for weak bases and for amphoteric electrolytes and may also be applied to strong electrolytes.